Understanding the biology of two commercially important crustaceans in relation to fisheries and anthropogenic impacts

Kevin Scott

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Heriot Watt University

The School of Energy, Geoscience, Infrastructure and Society (EGIS)

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ABSTRACT

The edible crab, *Cancer pagurus* and the European lobster, *Homarus gammarus*, are two commercially and ecologically important species found throughout Western Europe. Despite their importance there remains large knowledge gaps about both species, particularly around the effects of recent anthropogenic marine stressors such as unavoidable electromagnetic field (EMF) emissions from Marine Renewable Energy Devices (MREDs). Given the life history of *C. pagurus* and *H. gammarus* there is a high likelihood that they will come in to contact with MREDs and their associated power cables. To fully assess the potential impacts of EMF on *C. pagurus* and *H. gammarus* several factors were assessed including (1) behavioural aspects such as attraction/avoidance, activity level, and antennular flicking rate, (2) physiological aspects using commonly utilised crustacean stress markers, and (3) developmental aspects via examination of egg development, egg and larval morphology, and larval locomotory ability. Results were compared to two separate studies conducted to expand the knowledge on baseline stress markers for each species. Results confirm that EMF exposure negatively impacts both species on a behavioural, physiological and developmental level with far reaching implications. Results are discussed collectively and implications for management and future research suggested.

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Chapter 1. General Introduction

1.1 Ecology, habitat and distribution of *Cancer pagurus*

The edible crab, *Cancer pagurus*, has been one of the most important species in Western European fisheries for several centuries (Edwards, 1979). Identified by its characteristically heavy oval shaped body with a light brown 'pie crust' edge to the carapace, it is a common sight along rocky shores throughout the UK. Individuals typically have a carapace width (CW) of up to 250mm although larger males have been found with CW up to 300mm weighing up to 4kg (Figure 1.1.) (Neal and Wilson, 2008). Sexual dimorphism occurs within this species with males being larger and having considerably larger chelae than the females. Like many crustaceans female *C. pagurus* have a larger telson, utilised in mating and subsequent egg protection and larval release (Edwards, 1979).

Figure 1.1. 'Popeye' the crab (300mm CW, 4kg) caught in the English Channel pictured next to a typical female brown crab. Photo: Phil Yeomans BNPS.

C. pagurus is typically found throughout western Europe from Norway to Portugal, however sightings have been registered as far south as Angola in the South Atlantic Ocean (Ocean Biogeographical Information System, 2019). Found in the Atlantic Ocean, North Sea, Norwegian Sea, Tyrrhenian Sea, Mediterranean Sea (Koukouras *et al.*, 1992; Froglia, 2010; Marco-Herrero *et al.*, 2015), Adriatic Sea and thought to inhabit the Black Sea (Anosov, 2000) it is a highly prevalent species. This species inhabits a wide variety of habitats including bedrock, boulders, mixed coarse ground, muddy sand and kelp holdfasts (Moore, 1973; Eriksen and Moen, 1993; Robinson and Tully, 2000; Neal and Wilson, 2008; Heraghty, 2013; Scott *et al.*, 2018b) on the lower shore, intertidal shallow sublittoral and offshore to depths of around 400m (Bakke *et al.*, 2019) although most commonly found at depths around 100m (Neal and Wilson, 2008). Being a stenothermic species, they typically inhabit a narrow temperature range of 4 - 15°C (Cuculescu *et al.*, 1998). Salinity tolerances vary by age with juveniles (50 – 100cm CW), who occupy the intertidal zone (Williamson, 1900; Pearson, 1908), able to tolerate reduced salinities for extended periods of time compared to adults (17 psu) (Wanson *et al.*, 1983). A predatory species, this crab has been known to feed on a variety of prey items including gastropods (Lawton and Hughes, 1985), bivalves (Hall *et al.*, 1991; Mascaro and Seed, 2001), polychaetes (Lawton, 1989; Eriksen and Moen, 1993), echinoderms (Norderhaug *et al.*, 2014) and have been confirmed to prey on smaller conspecifics (Lawton, 1989). Typically, an ambush predator (Lawton, 1989), other methods of predation include pit digging for benthic organisms (Hall et al., 1991) and scavenging on carrion on the sea bed (Scott *et al.*, 2018a).

Characteristically, *C. pagurus* exhibit a crepuscular activity cycle, whereby during the daytime activity levels are significantly reduced and much of their time is spent hiding in shelters (Skajaa *et al.*, 1998; Scott *et al.*, 2018a; 2018b). This nocturnal behaviour is thought to occur to avoid predation by visually perceptive predators such as wolffish, birds, cod, octopus and seals (Rae 1967, 1968; Rae and Shelton, 1982; Boyle *et al.*, 1986; Skajaa *et al.*, 1998; Tallack, 2002).

C. pagurus are susceptible to a variety of bacteria, viruses, parasites, fungi, protists and metazoans (Stentiford, 2008). Black spot disease, which results from the breakdown of chitin by chitinolytic bacteria is prevalent across most fished crustaceans worldwide (Vogan *et al*., 2001), including in *C. pagurus*. In some areas there is the potential for

significant financial loss due to higher rates of rejection by the fishery and market as severely infected individuals produce poor quality meat (McIntosh, 1963). Previous studies have found some regional differences in disease for *C. pagurus*, although currently there is limited knowledge on the prevalence of different diseases within different populations, the extent of the mortality due to these diseases (Stentiford, 2008), the true economic cost of diseased individuals to the fishery, and the ecological effects on reproduction. Exposure to stressors has been shown to result in reduced immunocompetence, resulting in higher disease rates (Evans, 1999).

1.2 *Cancer pagurus* fishery and management

C. pagurus is the most valuable crab fishery (Haig *et al.*, 2015) and third most important shellfish fishery in the UK (Haig *et al.*, 2016) worth £44.6million in 2014, and £13.8million in 2013 in Scotland alone (Marine Scotland, 2017). FAO (Food and Agricultural Organization of the United Nations) statistics report an increased catch of *C. pagurus* around the UK and Ireland from 10,000 t in 1950 to 29,793 t in 2014 (Bannister, 2009; The Scottish Government, 2015). In 2014, a total of 32,500 t was landed in the UK by local and foreign vessels (MMO, 2015) with a further 5900 t in France, 7100 t in Ireland and 4692 in Norway (ICES, 2013). Global capture of this species peaked at 59,000 t in 2007 (FAO, 2019a).

Although there are no exact records of the early fishery of crabs within the UK crab fishermen from Devon can trace the tradition of crab fishing back to the Magna Carta (1215 AD) (Blyth *et al.*, 2002). Furthermore, the Roman word '*carabus*' most likely refers to *C. pagurus* (Edwards, 1979) and it has been suggested that crab fishing occurred during the time period when Romans occupied the southern part of the country (Bell, 1853; Lord, 1867) around 50 AD. There are also passing references to crab fishing boats from the 12th century in the Muster Rolls of Whitby Abbey, Yorkshire, UK (Edwards, 1979). The *C. pagurus* fishery is one of the largest crustacean fisheries in Western Europe, second only to Norway lobster, *Nephrops norvegicus*, which was worth £78.3 million in 2009 to Scotland alone (The Scottish Government, 2019a). Crabs are typically caught in coastal waters from May until October (Brown and Bennett, 1980; Mill *et al.*, 2009; Aitken, 2018) on small vessels (<14m) using baited traps with designs varying

around the coast (Figure 1.2.). Larger vessels targeting *C. pagurus* typically extend both fishing season (year-round) and fished area (offshore) (Mill *et al.*, 2009; Aitken, 2018). *C. pagurus* also appears as bycatch in large numbers in dredges (Ondes *et al.*, 2016).

Figure 1.2. Crab and lobster creels in St Abbs harbour, UK. Photo: Kevin Scott.

In the early 1800s the going price for a small crab (<104mm), typically used as bait but also for consumption, on the Norfolk coast was between $1\frac{1}{2}$d and $2\frac{1}{2}$d. The increase in sales of small crabs in the mid 1800s was a cause for concern, resulting in the creation of the *1876 Crab and Lobster Fisheries (Norfolk) Act* which prohibited the sale of crabs with a carapace width below 108mm and the landing of berried hens (Edwards, 1979). In this era, most crabs landed were transported for sale to Billingsgate Market, London via steamers and later by railway. After studies were conducted at 27 of the main fishing areas at the time around the UK and Ireland by Francis Buckland and Spencer Walpole, the information obtained resulted in the passing of the *1877 Fisheries (Oysters, Crabs and Lobsters) Act* which applied to the whole of the UK and made it illegal to land crabs under 108mm carapace width, berried hens and soft shell crabs. The same bill also prohibited landing of lobsters under 203mm total length and provided enforcement of the above Act by means of local closed seasons for crabs and lobsters in any area within the

UK (Edwards, 1979). It was after the passing of this Act that fishermen around the UK first started recording catch and sales aboard their vessels (Edwards, 1979). Further revisions of this Act in 1894 (*Sea Fisheries Regulation Act*), 1951 (*Sea Fishing Industry (Crabs and Lobsters) Order*), 1966 (*Sea Fishing Industry (Crabs and Lobsters) Order*), 1976 (*Immature Crabs and Lobsters Order*) saw increases in crab carapace width to 115mm. Since 1976 greater emphasis has been placed on regional changes throughout the UK resulting in different Minimum Landing Sizes (MLS) for crabs and lobsters by geographic regions. In Scotland the MLS for *C. pagurus* is 150mm (140mm in Shetland) (The Scottish Government, 2018). English fisheries enforce a slightly different set of regulations with 140mm MLS for *C. pagurus*, with regional differences ranging from 130mm (south of 56°N) to 160mm (Devon, Cornwall and Scilly Isles) (Marine Management Organisation, 2018; North East Inshore Fisheries and Conservation Authority, 2019). To date, *C. pagurus* is not a quota species throughout its distribution (Haig *et al.*, 2016).

1.3 Life history of *Cancer pagurus*

The basis of the literature on the life history of *C. pagurus* stems from several studies on this topic conducted in the late 1800s – early 1900s. Works by Cunningham (1898), Williamson (1900, 1904), Pearson (1908), Meek (1912, 1914, 1916) and later Bjerkan (1927) took great steps in expanding our understanding of this species' early life history, migrations, and breeding behaviour with studies by Williamson and Meek taking place within the same section of coast where this study took place.

It is well established that mating within this species occurs between a recently moulted soft female and a hard carapace male (Bennet, 1995) typically around December to February (Pearson, 1908) although varies year to year and by location (Bennett, 1995). Recent studies by Haig *et al.,* (2016) surmised that the carapace length at first maturity ranged from 59 – 106mm in males and 97 – 117mm in females. Sexually mature male and females will pair up prior to copulation (for 3 – 21 days), with the male aiding in ecdysis of the females by providing protection and physically removing the old exoskeleton (Edwards, 1979; Haig *et al.*, 2016). During copulation the male rotates the female on to her back and deposits sperm into the female's vulva (Williamson 1900;

Edwards, 1979). After copulation, females' genital openings are blocked via sperm plugs, which are visible several weeks after mating (Edwards, 1979), thus prohibiting any further mating (Williamson, 1904; Edwards, 1966). During the mating season, it has been shown that some males are polygamous and may attend several females leading to multiple successful matings (Edwards, 1979). After mating it has been hypothesised that many females spend up to a year replenishing energy stores, which aids in carapace recalcification, before spawning (Pearson, 1908; Edwards, 1979). Males crabs have been shown to migrate shorter distances than female crabs (Ungfors *et al.,* 2007) throughout the year. However, females have been shown to undertake large migrations, typically to deeper water offshore, during the spawning season (Bennett and Brown, 1983; Ungfors *et al.,* 2007; Hunter *et al.,* 2013). Female crabs typically select a soft sandy substrate into which they dig a pit using their chelae (Hall *et al.,* 1993). Females then settle their telson into the pit to provide shelter from currents and passing predators (Brown and Bennett, 1980), before oviposition occurs. Oviposition takes around 12 – 24 hours for egg adhesion to the pleopods (Edwards, 1971). Females can lay anywhere between 460,000 – 3,880,000 eggs (Williamson, 1900; Edwards, 1967; Tallack, 2007; Ungfors, 2007; Haig *et al.,* 2015). Once oviposition is complete, berried females spend around 6 – 9 months buried, with minimal movement and lower feeding rates (Williamson, 1900; Edwards, 1979; Howard, 1982; Naylor *et al.,* 1997). Females will adjust the frequency of egg ventilation as oxygen demand increases during egg development (Naylor *et al.,* 1999). During this stage berried females are rarely found in baited traps due to lack of movement and reduced feeding rates, however they are found as bycatch in scallop dredgers in the Isle of Man, subsequently suffering high damage and mortality rates (Ondes *et al.,* 2016). During periods of high stress, berried hens have been shown to drop their eggs, consume their own eggs, or consume the eggs of other females (Haig *et al.,* 2015). When larvae are close to hatching, berried females have been shown to undertake large migrations in order to take advantage of locally occurring currents which aid in larval dispersal (Ungfors *et al.,* 2007). After around 8 months when the eggs have reached full development, they will begin to hatch as protozoea larvae before progressing through 5 zoeal stages resulting in the final larval stage known as megalopa. Once megalopa the larvae will begin to settle from the water column to the benthos within 24 hours (Edwards, 1979). Larval release has been shown to coincide with spring plankton blooms during the warmer spring and summer months (Haig *et al.,* 2015). After females have finished

releasing larvae they have been known to migrate inshore to forage and replenish energy reserves (Howard, 1982).

Despite the high value of this commercially important species there remains significant gaps in terms of understanding basic population factors (e.g. age, size at maturity, fecundity) (Haig *et al.*, 2016) and early life history, particularly post-settlement.

1.4 Ecology, habitat and distribution of *Homarus gammarus*

The European lobster, *Homarus gammarus*, is a large commercially important decapod (Figure 1.3.) which inhabits a very similar geographic region to *C. pagurus*. Found throughout much of the eastern North Atlantic from Morocco to Norway they have been found in the North Sea, Mediterranean Sea, Adriatic Sea, Ionian Sea and the Black Sea (Wilson, 2008). Unlike other species of lobster, and many species of crustaceans in general, they do not move great distances, rarely venturing more than a few hundred metres from their home range (Moland *et al.*, 2011; Skerritt *et al.*, 2015). *H. gammarus* can grow up to 650mm in total length (Wolff, 1978) although they are typically found around 50cm in length with large distinguishable chelae (Wilson, 2008). Inhabiting a variety of habitats including bedrock, rocky substrata and boulders (Smith *et al.*, 1999; Mehrtens *et al.*, 2005; Galparsoro *et al.*, 2009) they are typically found to depths of around 60m (Wilson, 2008) although have been recorded as deep as 150m (Holthius, 1991; Irish Sea Fisheries Board, 2015). Salinity tolerances are relatively high in *H. gammarus* with temporary salinities as low as 10ppt reporting no lasting damage (Jury *et al.*, 1994), however young lobsters and larvae have been found to be more susceptible to lower salinities (Charmantier *et al.*, 2001). Adults occupy a temperature range of 0 – 25°C which coincides with their distributional range (Qadri *et al.*, 2007). Temperatures of up to 20°C have been reported during egg incubation and larval development and subsequently showed no adverse effects which suggests a wide temperature range for this species (Gruffydd *et al.*, 1975; Branford, 1978). Previous studies have shown detrimental effects of sudden temperature changes such as exposure to fluctuating air temperatures on boats after catch, which results in lobsters becoming hypoxaemic, hypercapnic and showing increased lactate concentrations in haemolymph (Whiteley *et al.*, 1990).

Figure 1.3. Female European lobster, *Homarus gammarus*. Photo: St Abbs Marine Station.

H. gammarus, like *C. pagurus*, show increased activity levels during dawn and dusk (Smith *et al.*, 1999; Moland *et al.*, 2011). This increase in activity has been linked to a photoperiod circadian rhythm (Smith *et al.*, 1998; 1999; Jury *et al.*, 2005) which results in a diel activity pattern previously shown to benefit lobsters by reducing susceptibility to predation by visual based predators during daylight (Smith *et al.*, 1998; Moland *et al.*, 2011). A negative correlation has been found between lobster size and activity levels (Mehrtens *et al.*, 2005). During these periods of higher activity, foraging and hunting take place with lobsters preying on a variety of items including crabs, bivalves, worms, and molluscs (Wahle *et al.*, 2013). Lobsters have a highly developed sensory system which is used to detect prey movement under low light conditions, however they have been found to be slow, periodic feeders (Mente *et al.*, 2001).

Like *C. pagurus* lobsters are known to be affected by a variety of diseases of bacterial, viral, protistan, fungal, and parasitic origin (Cawthorn, 2011). Despite fewer reported pathogens and parasites relative to other decapod crustaceans (Behringer *et al.*, 2012) diseases such as gaffkaemia, bumper car disease and shell disease such as black spot are still prevalent (Ayres and Edwards, 1982; Cawthorn, 2011). Lobsters, despite their

commercial fishery importance, are integral members of their benthic communities where predatory-prey, competitive, and host-pathogen relationships can significantly affect their population dynamics (Behringer *et al.,* 2012).

1.5 *Homarus gammarus* fishery and management

H. gammarus fisheries are some of the most lucrative in terms of price per unit worldwide. Landings of lobster by Scottish vessels has increased substantially in recent years from 290 t in 2001 to 940 t in 2009 with a value of £11.4million (The Scottish Government, 2019b). A substantial increase in total landings worldwide has seen increases from 3,000 t in 1950 to 4,713 t in 2016, with a peak of around 5,500 t in 2011 (FAO, 2019b).

Figure 1.4. Crab and lobster creels in St Abbs harbour, UK. Photo: Kevin Scott.

Certain countries, such as Norway, have seen significant declines in catch by around 65% with today's catch being the lowest on record (Pettersen *et al.,* 2009). The reasons behind Norway's decline in lobster catch has been hypothesised to be due to an increase in fishing pressure, ineffective regulations, and unreported catches (Agnalt *et al.,* 1999; Pettersen *et al.,* 2009) with some estimates putting total catch 14 times higher than those on record

(Kleiven *et al.,* 2012). Current regulations imposed for *H. gammarus* in Scotland are MLS 87mm CW (90mm in Shetland, Orkney, Outer Hebrides, Cape Wrath to 55°N), maximum landing size of 145mm (155mm in Shetland) for females, and it is currently prohibited to land crippled females (missing one or both claws) in the inshore waters of the Outer Hebrides, and individuals bearing a V-notch (The Scottish Government, 2018). In England an MLS exists of 87mm and a ban on landing berried hens (North East Inshore Fisheries and Conservation Authority, 2019). Lobsters are caught using baited traps (Figure 1.4.) by mostly small (<14m) vessels working inshore waters with most lobsters caught in water shallower than 30m (The Scottish Government, 2019b). The fishing season for lobsters runs from April to October with peak numbers typically caught between April and June then August to October.

1.6 Life history of *Homarus gammarus*

Estimates of size at sexual maturity varies by means of physiological, morphological and functional indices of maturation (Wahle *et al.,* 2013). On the east coast of Scotland Lizarraga-Cubedo *et al.* (2003) estimated that size at maturity for this population of *H. gammarus* was between 79 – 80mm CL based on morphological growth between the body and claws. An additional study in Ireland found size at sexual maturity to be around 92-96mm (Tully *et al.,* 2001). Mating typically occurs during the summer months post ecdysis with mature female lobsters choosing their partner (Debuse *et al.,* 2003). Female lobsters have been shown to visit nearby male's shelters, presumably attracted by the male's scent (Atema and Cowan, 1986). Mating follows the same routine as for *C. pagurus* with females moulting, being flipped on to their back and males passing sperm packets, of which several clutches can be fertilised (Aiken and Waddy, 1986). Females will then transport the sperm past the hardened sperm plugs for external fertilisation of the eggs which are attached to the pleopods (Aiken *et al.,* 2004). Female *H. gammarus* can also mate during the intermoult phase which further highlights the flexibility of lobster reproduction (Aiken and Waddy, 1986; Comeau and Savoie, 2002). Females have been shown to lay up to 14,000 eggs per clutch (Lizarraga-Cubedo *et al.,* 2003). Eggs are incubated for a period of 9 – 11 months before larval hatching the following summer (Agnalt *et al.,* 2007). Larval hatching typically occurs during the night, lasting for successive nights for periods of 2 – 6 weeks (Ennis, 1973).

Larvae have a pelagic phase (zoea I – IV) of around 6-8 weeks pre-settlement with potential to travel extensive distances along ocean currents (Huserbraten *et al.,* 2013). Subsequent studies, based on currently available technology, confirm that despite, large dispersal differences there are no spatial genetic structures throughout the lobster range with only weak differentiation between populations within the Swedish Skagerrak and the Atlantic areas to the West (Ellis *et al.,* 2017). During stage IV larvae begin to transform from a pelagic existence to life on the benthos.

1.7 Renewable energy in the marine environment

Crab and lobster habitat is currently subject to competition for space with the need for Marine Renewable Energy Devices in coastal waters. Due to the significant increases in greenhouse gas (GHG) emissions over the last several decades, environmental pollution has become one of the major worldwide issues (Dogan and Seker, 2016). Anthropogenically induced climate change through the burning of fossil fuels has a significant evidence base, which has led to many governments initiating programs for increased production of renewable or 'green' energy (Inger *et al.,* 2009). The world's coal, gas, and oil resources are finite, and consumption worldwide continues to increase (Greene *et al.,* 2004) and is predicted to do so as the world's population increases (Mackay, 2008). With the predicted decrease in non-renewable energy sources in future decades (Pimentel *et al*., 2002) there comes an increasing need for renewable sources to fill this void. Marine Renewable Energy (MRE) promises to assist by providing clean, inexhaustible energy and aid in the reduction of GHG emissions (Boehlert and Gill, 2010). The rapid increase in renewable energy in the relatively untapped marine environment (Inger *et al.,* 2009), is not without its problems. There are social and environmental concerns including habitat loss, collision risks, increased anthropogenic noise, exposure to increased electromagnetic fields (EMF) (Pelc and Fujita, 2002; Gill, 2005; Cada *et al.,* 2007; Boehlert *et al.,* 2008 Inger *et al.,* 2009; Scott *et al.,* 2018a). There are three prominent types of marine renewable energy: wave, tidal, and wind. Wave Energy Converters (WEC) rely on wind waves to generate electricity by pushing hydraulic fluid through electrical generators (attenuator operated on surface e.g. PELAMIS), compressing air through air turbines (oscillating water column, found on shore and in deeper waters), or by having floating buoys attached to the seabed acting as

a point absorber which generates electricity by using the rise and fall of the waves to power linear generators (point absorber buoy, connected to sea bed with floating buoy on the water surface) (Alternative Energy Tutorials, 2017). The first patent for a WEC was filed in 1799 (Clement *et al.,* 2002), since then numerous patents have been filed, yet it is not widely employed commercially (Miller, 2004) despite significant testing taking place at the European Marine Energy Centre (EMEC) in Orkney, UK. Tidal energy devices utilise reliable and predictable tidal movements of water to power underwater turbines. Tidal energy is one of the oldest forms of renewable energy dating back to the Roman times (Minchinton, 1979) when water movement was utilised to mill grains. Tidal energy is again not widely employed commercially throughout the UK with many developments in the testing phase. With tidal turbines there comes an increased risk of marine life colliding with the underwater blades. The tidal turbines in Strangford Lough, Northern Ireland, are equipped with sensors that turns the turbines off when marine animals approach, although this has led to a major loss in energy production due to the extensive periods that the turbines are non-operational (Kempener and Neumann, 2014).

Wind energy, the harnessing of wind via turbines, is by far the most abundant form of renewable energy in the UK with 1,500 onshore turbine arrays (12 GW) producing 9% of the UK's power needs in 2017, with a cumulative investment of over £35 billion (Renewable UK, 2019). Due to planning restrictions, lack of inexpensive land near population centres (Bilgili *et al*., 2011) and aesthetic problems (Gill, 2005), renewable energy structures are increasingly being located offshore. The significant potential for generating energy offshore has led to an increase in offshore renewable infrastructure (Muller, 2013). Currently, the UK is the largest global producer of electricity from offshore wind farms and has more projects in planning or construction than any other country worldwide (Smith *et al.*, 1999; The Crown Estates, 2016).

Offshore wind in the UK powers the equivalent of 4.5 million homes annually with the aim of generating 10% of all UK electricity by 2020, through £19 billion investment between 2016 - 2021 (Renewable UK, 2019). Wind energy has one of the lowest levelized cost per MWh of electricity generation when a carbon cost is included in electricity generation (Department for Business, Energy & Industrial Strategy, 2016), cheaper than new gas and nuclear power (Renewable UK, 2019).

Proposed sites are based on the current state of the art, which include the information provided from local environmental assessments and from scientific literature detailing suggestions on the effects of renewable energy on various habitats. However, significant gaps exist in the current knowledge of the effects of renewables on marine and freshwater organisms (Cada *et al.*, 2011; Scott *et al*., 2018a; Dannheim *et al.*, 2019). All renewable energy devices have one thing in common: power cables. Marine Renewable Energy Devices (MREDs) are currently connected via sub-sea power cables, inter-turbine cables, cables to power storage banks, and export cables from deployment site to shore (Figure 1.5.). These sub-sea power cables, like most current carrying cables, emit electromagnetic fields (EMF).

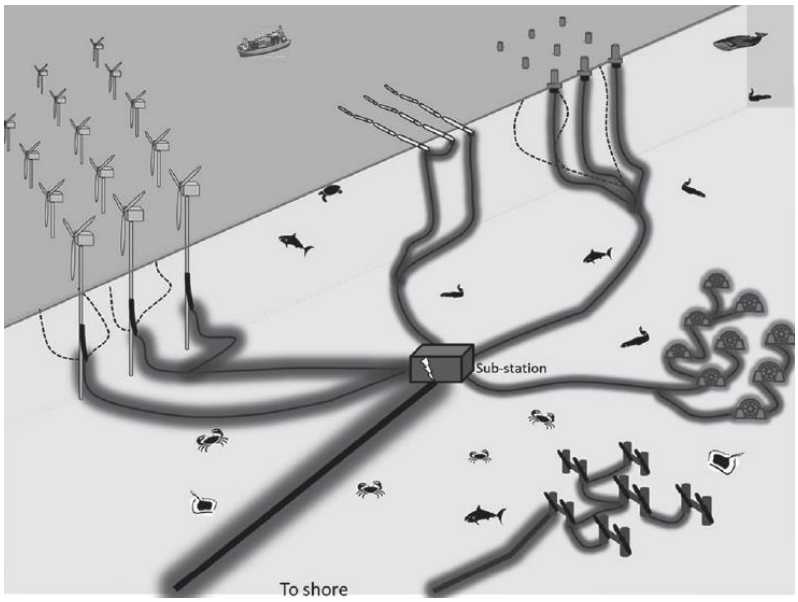


Figure 1.5. Diagram from Gill *et al.* (2014) showing a simplified version of cable configurations and types around a variety of MREDs with EMF emissions noted by the dark shading around cables.

Previous studies have shown that there is not currently an industry standard insulation that can successfully shield from both electric field (E-field) and magnetic field (B-field) emissions (Gill, 2005). The result of this B-field leakage combined with standard cable configurations results in an induced EMF (iEMF) (Gill, 2005) that could potentially cover large areas of the benthos around the deployment boundaries, depending on the scale and

the number of cables used in a certain area. The resultant iEMF will be affected by saltwater ion movement via underwater currents near cables, the current type and strength passing through, which will result in an EMF of variable size and strengths that are extremely hard to predict. Normandeau *et al.* (2011) reported a great variation of EMF strengths around different structures associated with MREDs, whilst Thomsen *et al.*, (2015) reported higher strengths around export cables, which in recent years utilise High Voltage Direct Current (HVDC) cables, rather than inter-device cables typically consisting of Alternating Current (AC). A review of the literature (CMACS, 2003) indicates that the current knowledge on EMF strengths emitted from MREDs is insufficient to allow for an informed assessment. EMF strength is measured in Tesla (T) and subsequent derivations thereof with 1T = 1000mT = 1 x 10^6µT. EMFs also originate from natural sources such as the Earth's natural magnetic field (Scott *et al.,* 2018a) where field strengths of between 0.03mT and 0.06mT occur with variations due to the Earths polar regions and storms. EMF strengths reported in the literature vary from 0.14mT – 8.02mT (Bochert and Zettler, 2006; Normadeau *et al.*, 2011; Cada *et al.*, 2011) with values used in scientific studies ranging from 2.8 – 165mT (Formicki *et al.*, 2004; Bochert and Zettler, 2004; Cada *et al.,* 2011; Woodruff *et al.*, 2012; Scott *et al.*, 2018a). Currents of around 450 to 1600 Amperes (A) (AC 715A (33kV cable) – 525A (220kV cable) (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019) are found in undersea power cables resulting in EMF emissions of 3.2mT in a perfect wire (Bochert and Zettler, 2006). It has been shown in models that the strength of an EMF diminishes the further it travels from the source cable (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019) , whereby 220kV cables produce a 22µT magnetic field at source that falls to 2µT 5m away from the cable. Similarly, a 33kV cable produces a 13µT field that falls to 0.5/1 µT 5m away from the source (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019). Bochert and Zettler, (2006) also highlighted the change in a 1600A cable diminishing from 3.2mT at the cable surface to 0.32mT and 0.11mT at 1m and 4m respectively. The unpredictability, and difficulty in assessing the true EMF, particularly the iEMF, around MREDs makes it difficult to determine the potential effects on marine life. Effects of increased EMF exposure have been found in fish including: changes in melatonin synthesis in brook trout, *Salvelinus fontinalis*, (Lerchl *et al.,* 1998), behavioural changes in trout, *Salmo trutta*, (Formicki *et al.*, 2004), variations in larval rearing in

European sheatfish, *Silurus glanis*, (Krzemieniewski *et al.*, 2004), and changes in migration behaviour in European eel, *Anguilla anguilla*, (Westerberg and Lagenfelt, 2008). Effects have also been discovered in birds including: turbine collisions in seabirds (Garthe and Huppop, 2004), and changes in spatial distribution of Loons, *Gavia* sp., (Mendel *et al.*, 2019). Ecological Impact Assessments for MREDs are based upon current scientific knowledge which is significantly lacking regarding the effects of EMF on marine invertebrates. Given the ecological and financial importance of *C. pagurus* and *H. gammarus* to the UK and its surrounding waters further work must be conducted to determine what effects these increased levels of EMF will have on their behaviour, physiology, and development. This information is essential for the future management and mitigation of problems around MRED sites.

Scour protection zones set up around the base of MREDs to reduce erosion also act as artificial reefs and subsequently increase habitat by as much as 2.5 times than that lost by array installation (Linley *et al.*, 2009). Previous studies suggest offshore renewables and their associated scour protection areas may enhance biodiversity through new habitat creation (Landers Jr *et al*., 2001; Lindeboom *et al*., 2011). In a study by Kawasaki *et al.* (2003) it was suggested that if the habitat requirements for specific target species were considered during MRED scour zone and artificial reef construction then the abundance and diversity of associated species could be enhanced. The addition of scour protection zones combined with the many no-take zones around turbine arrays will enhance overall crustacean populations (Langhamer and Wilhelmsson, 2009).

Langhamer and Wilhelmsson (2009) conducted a study at the *Lysekil Project* test park off the Swedish coast investigating the effects of drilling various sized holes into the scour protection blocks. The results showed that a significantly higher abundance of fish and crabs were present on the foundations compared to the surrounding soft bottoms. A five-fold increase was seen in the number of *C. pagurus* present when holes were drilled into the scour zones. An increase in *C. pagurus* was also found in studies by Krone *et al.* (2013, 2017) who showed that double the *C. pagurus* numbers are present on turbines with scour protection zones, 27% of the local *C. pagurus* stock will come from these areas and that a predicted 60 – 165% increase in substrata-limited mobile demersal species will occur in these areas. This increased capacity for benthic and pelagic organisms, combined with the no-take fishery zones (typically 50m from turbines) commonly implemented

around offshore windfarms is expected to cause an increased spill-over effect with nearby fished areas predicted to see an increase in catch (Punt *et al.,* 2009). However, further research is needed to determine what impact the deployments will have on these species.

Analysis of haemolymph is a common method for measuring stress in crustaceans (e.g. Hagerman, 1986; Lorenzon *et al*., 2011; Scott *et al*., 2018a; Ooi *et al.,* 2019). Haemocyanin, the primary oxygen carrying protein in crustaceans, has been shown to increase during periods of hypoxia (Hagerman *et al.,* 1990) and has been used in previous experiments to assess stress levels (Hagerman, 1983, 1986). Total Haemocyte Counts (THCs) are also used as a measure of stress (Durliat and Vranckx, 1983; Persson *et al*., 1987; Smith *et al.,* 1995; Jussila *et al.,* 1997; Lorenzon *et al.,* 2007). Haemocytes play important roles in immune responses of crustaceans (Johansson *et al.,* 2000). There are several classifications of haemocyte cells including: hyaline cells (involved in phagocytosis), semi-granular cells (involved in encapsulation), and granular cells (involved in the release of prophenoloxidase) (Johansson *et al.,* 2000). D-Glucose is the primary fuel for Adenosine Triphosphate (ATP) production in crustaceans and is essential in maintaining metabolic processes (Verri *et al.,* 2001; Jimenez and Kinsey, 2015). D-Glucose concentrations have been shown to rise continually in relation to increased locomotor activity (Hamann, 1974; Reddy *et al.,* 1981; Kallen *et al*., 1988; Kallen *et al*., 1990; Tilden *et al.,* 2001), as well as in response to stress such as emersion (Morris *et al*., 1986; Taylor *et al.,* 1997; Bergmann *et al*., 2001; Speed *et al.,* 2001; Lorenzon *et al.,* 2007), pollutants (Nagabhushanam and Kulkarni, 1981; Machale *et al.,* 1989; Reddy and Bhagyalakshmi, 1994; Lorenzon *et al*., 2000), and disease (Lorenzon *et al*., 1997). L-Lactate, as a metabolite in crustaceans is typically an indicator of anaerobic respiration due to hypoxic/anoxic conditions or impaired gill function (Durand *et al.,* 2000). L-Lactate concentrations in crustacean haemolymph has also been shown to increase during exposure to various stressors (Spicer *et al.,* 1990; Taylor *et al.,* 1997; Durand *et al.,* 2000; Bergmann *et al*., 2001; Ocampo, Patiño and Ramírez, 2003; Lorenzon *et al.,* 2007; Scott *et al*., 2018a).

In addition to physiological analysis, behavioural assessment is also frequently used to determine the effects of various stressors (Scott *et al.,* 2018a). Behavioural and response parameters (attraction/avoidance, antennular flicking rate, and activity level) can be

affected by various environmental stressors such as temperature, salinities, sound and EMF exposure (Stoner, 2012; Scott *et al.*, 2018a, 2018b).

1.8 Project Aims

There is a clear need for evidence-based information regarding the potential effects of increased EMF exposure on both *H. gammarus* and *C. pagurus* to inform of potential threats to fisheries, ecological interactions and stock recruitment of these important species. In order to fully obtain information regarding the effects of EMF exposure, we first need to obtain solid baseline data on the commonly used crustacean stress markers which is currently lacking for both species. Data on behavioural (including activity levels, shelter selection and antennular flicking rate for *C. pagurus*) and physiological (Haemocyanin, L-Lactate, D-Glucose, Total Haemocyte Counts and respiration for *C. pagurus*) parameters will be collected to inform for future stress-based studies. Once baseline data has been obtained for both species, a device which can recreate EMF homogenously at the predicted strengths of EMF around power cables will be created. Using several Helmholtz coils, and solenoid electromagnets, working at a variety of strengths, the impacts of exposure to increased EMF will be measured and compared to the baseline data previously obtained. Given the inconsistencies in data reporting the strengths of EMF, and in order to provide a tool which can be used for future risk assessment and management, an additional study will be conducted on *C. pagurus* using several EMF strengths. Given the life cycle of both species, the long periods spent brooding eggs, and the high probability of coming in to contact with subsea power cables, information is required on the effects of EMF exposure on egg development and subsequent larval locomotory ability. To determine these effects, berried female *C. pagurus* and *H. gammarus* will be subjected to increased EMF exposure throughout egg development and larval hatching with continuous measurement throughout that time period.

1.9 Thesis Structure

The following chapters (2 – 10) are set out to address the key issues listed above. A brief overview of each chapter is provided below:

Chapter 2 provides baseline data for *C. pagurus* on commonly used behavioural and physiological stress markers in crustaceans. Aiming to fill a significant gap in the literature this chapter assesses activity level, antennular flicking rate, Haemocyanin, L-Lactate and D-Glucose concentrations, haemolymph density, and respiration over 24 h.

This chapter is published in the *Journal of Experimental Marine Biology and Ecology*:
Scott, K., Harsanyi, P. and Lyndon, A.R., 2018. Baseline measurements of physiological and behavioural stress markers in the commercially important decapod *Cancer pagurus* (L.). *Journal of Experimental Marine Biology and Ecology*, *507*, pp.1-7.

Chapter 3 provides comparable baseline data on commonly used crustacean stress markers for *H. gammarus*. Building upon available data for the closely related *Homarus americanus* this chapter obtains valuable data on activity level, haemolymph density, Haemocyanin concentrations, Total Haemocyte Count, L-Lactate and D-Glucose concentrations over a 24 h period.

This chapter has been submitted to *Crustaceana* with the following authors: Scott, K., Harsanyi, P., de la Cruz, G., Easton, B., Chapman, E.C.N., and Lyndon, A.R.

Chapter 4 examines the effects of exposure to increased EMF (2.8mT and 40mT) on juvenile and adult crabs by assessing behavioural (attraction/avoidance, activity level, shelter selection, antennular flicking rate) and physiological (Haemocyanin, L-Lactate, D-Glucose, respiration) parameters.

This chapter is published in *Marine Pollution Bulletin*:
Scott, K., Harsanyi, P. and Lyndon, A.R., 2018. Understanding the effects of electromagnetic field emissions from Marine Renewable Energy Devices (MREDs) on the commercially important edible crab, *Cancer pagurus* (L.). *Marine Pollution Bulletin*, *131*, pp.580-588.

Chapter 5 furthers the research on the effects of EMF on commercially important crustaceans by assessing *H. gammarus* at 2.8mT. During exposure measurements were conducted on activity level, time spent resting/roaming, shelter selection, Total Haemocyte Count, D-Glucose, and L-Lactate concentrations over 24 h.

Chapter 6 assesses the impacts on egg incubation and subsequent larval locomotory ability of both *C. pagurus* and *H. gammarus* during exposure to increased EMF (2.8mT). By undertaking measurements throughout egg development of exposed and control animals including egg morphometrics, weights and stages, the impacts of females incubating eggs near subsea power cables was determined. Upon hatching larvae were subjected to movement tests to assess potential effects of EMF on larval locomotory ability.

Chapter 7 continues examination of the effects of EMF exposure on *C. pagurus* but includes re-assessment under different EMF strengths. Parameters found to be affected by EMF exposure during Chapter 4 were again utilised with the aim of providing data that would prove invaluable to risk assessments and EIAs.

Chapter 8 summarises key findings throughout this study and discusses how findings may be applied to future MRED management. Future research is discussed in addition to highlighting the remaining knowledge gaps.

Chapter 2. Baseline measurements of physiological and behavioural stress markers in the commercially important decapod *Cancer pagurus* (L.)

2.1 Introduction

It is generally accepted that there are increasing stresses on the marine environment (Crain *et al.,* 2008; Gunderson *et al.,* 2016), including many of anthropogenic origin, such as plastic waste, noise, excess nutrients, thermal effluents, pollutants, acidification and electromagnetic fields (Scott *et al.,* 2018a), amongst others. Consequently, there are likely to be negative impacts on marine organisms manifested through stress responses (Chang, 2005), which may ultimately affect organism fitness (Calow and Forbes, 1998). Stress responses in crustaceans have been sporadically investigated over the last 20 years, generally in relation to transport of commercial species (Taylor *et al.*, 1997; Lorenzon *et al.*, 2008; Barrento *et al.*, 2010, 2011). However, there remains a dearth of baseline data on stress responses relevant to environmental stressors, particularly in relation to behavioural correlates of stress.

The European edible crab or brown crab (*Cancer pagurus* L.) is a commercially important brachyuran decapod, being exploited throughout Western Europe, from Norway to northern France (Edwards, 1979; Karlsson and Christiansen, 1996). It was worth £13.8million in 2013 in Scotland alone (Marine Scotland, 2017) and is the most valuable crab fishery in UK waters (Haig *et al.,* 2015). FAO (Food and Agriculture Organization of the United Nations) statistics report increased landings of *C. pagurus*, around Britain and Ireland from 10,000t in 1950 to 29,793t in 2014 (Bannister, 2009; The Scottish Government, 2015).

Cancer pagurus are both active predators and scavengers, consuming a wide range of prey items, and are found from shorelines to depths of 90 m, with larger mature specimens being found offshore (Neal and Wilson, 2008). Predominantly they are found in the sub-littoral zone where they experience a relatively narrow seasonal temperature range of 4–15 °C (Cuculescu *et al.,* 1998). Characteristically, they exhibit a nocturnal activity cycle, whereby during daytime they tend to hide in shelters within bedrock or soft substrate with

reduced movement levels (Skajaa *et al.,* 1998; Scott *et al.,* 2018a). Their behavioural repertoire also includes sensing of the environment through flicking of their antennules, which have been shown to be involved in olfaction (Stensmyr *et al.,* 2005).

Several stimuli such as food, pheromones, predators, vibration and sound are known to elicit antennular flicking responses in several crab species (Keller *et al.,* 2003; Stensmyr *et al.,* 2005). Previous studies have utilized antennular flicking rates as a response to a stimulant and/ or stressor in hermit crab (Snow, 1975), spiny lobster (Daniel and Derby, 1991), American lobster (Berg *et al.,* 1992), crayfish (Mellon, 1997) and Dungeness crab (Woodruff *et al.,* 2013).

Respiration rates in marine organisms have been shown to be a reliable indicator of certain environmental stressors (Paterson and Spanoghe, 1997; Doney *et al.,* 2012; Brown *et al.,* 2013). In a paper by Bradford and Taylor (1982) it was demonstrated that *C. pagurus* has a high degree of respiratory independence in that, during hypoxic conditions they can maintain a constant oxygen consumption rate until an air saturation percentage of around 38% is achieved, after which their respiration rate dramatically decreases. However, there is a lack of information about juvenile edible crab respiration rates.

In crustaceans, haemolymph sampling, and its subsequent analysis, enables measurement of stress through detection of abnormalities in internal chemical processes. In previous studies (Taylor *et al.,* 1997; Durand *et al.,* 2000; Bergmann *et al.,* 2001; Lorenzon *et al.,* 2011) it was shown that L-Lactate, D-Glucose and haemolymph densities are useful components in measuring stress levels in crustaceans by highlighting changes in homeostasis.

The aims of the current work were, firstly, to establish baseline data for a variety of haemolymph markers in *C. pagurus* that have been widely used to measure stress responses in other crustaceans; secondly, to determine baseline data for antennular flicking rate, which is related to chemosensing and ventilation rates; and thirdly, to gain insight into diel individual activity levels via remote camera observation.

2.2 Materials and Methods

Intermoult crabs were obtained from local fisherman and via the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK). Prior to experimentation each crab was sexed, carapace width (CW; mm) measured and weighed (g). All crabs were categorized into CW size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+). Crabs chosen for experimentation had no damage to the carapace and were missing no more than two legs (i.e., classified as good or perfect condition based on Scott *et al.*'s, (2018a) condition index). Crabs were kept in a 1000 L flow through system at ambient sea temperature (range 13.7–14.5 °C) and natural photoperiod (range 12–14 hl) for a minimum acclimation period of 1 week and fed on frozen ragworm and live mussel during the acclimation period. Food was withheld for 24 h prior to experimentation. Experimentation was conducted July – August 2017.

Behavioural analysis

Activity level

Four 70 L tanks were connected to a 1000 L temperature-controlled sump tank which received a constant supply of UV sterilised, filtered sea water. Each tank was shaded along the sides to reduce visual disturbances. A wide aperture mesh was secured over the top of the tanks during the night to prevent the crabs from escaping. A submersible pump was used to pump water via a Hozelock adjustable control panel to the experimental tanks at an equal rate of around 3 L/min. A temperature and light pendant (Onset HOBO) were placed into each tank to monitor conditions. Individual crabs were placed into each experimental tank per trial and allowed to acclimate for 1 h before the start of experiment. After each trial the tanks were drained, sterilised (Virkon aquatic) and refilled.

Four waterproof InfraRed (IR) cameras (Sannce 1080p IR surveillance DVR system) were suspended above the experimental tanks and set to record during each trial. Trials consisted of:

1. Day conditions – 8 h (08:00 am-16:00 pm)

2. Night conditions – 8 h (20:00 pm-04:00 am)

Footage was then organised into images at every minute elapsed and analysed using Solomon Coder (version – beta 17.03.22). Activity level was then calculated as the percentage of 1-min intervals where movement occurred throughout each trial. A total of 92 individuals were analysed in day conditions (small = 26, medium = 20, large = 46) and 49 in night (small = 22, medium = 11, large = 34).

Flicking rate

A 12 L glass tank, containing a perforated plastic adjustable arena, was set up on a temperature controlled recirculation system, with a 40 L sump tank containing 45 μm filtered, UV sterilised seawater and an air stone. The inflow and outflow were separated from the test animal to reduce visual disturbance. The experimental tanks were behind opaque partitions to further reduce external stimuli.

Crabs were acclimated to experimental tanks for 30 min prior to testing after which the camera was set to record via a remote. The trials were recorded for a total of 10 min. The entire experimental system was sterilised and underwent a full water change after each trial. Temperature, dissolved oxygen and salinity were monitored before and after each trial.

The video data was post-processed with the flicking rate counted for both antennules, for each trial, then converted to average flicks per minute for each crab. Each video file was counted by 3 trained persons to ensure accuracy and consistency. A total of 10 individuals were used for each of the three size groups (N = 30).

Physiological analysis

Haemolymph analysis

Crabs were placed individually in temperature controlled (TECO TK1000), experimental tanks for a period of 24 h, and sampled at 0 h (9:00 am), 2 h (11:00 am, large crabs only), 4 h (13:00 pm), 6 h (15:00 pm, large crabs only), 8 h (17:00 pm) and 24 h (9:00 am). Samples were staggered with 5 min between each sample taken to

ensure consistency with sample times throughout the experiment. The sampling protocol used was:

Haemolymph samples were collected from the fifth walking leg using 1 ml syringes with 25G needles. To reduce handling stress this procedure did not take longer than 60 s. Approximately 250 µl, 300 µl and 700 µl were collected from the small, medium and large size groups respectively. The haemolymph was transferred into 1.5 ml cryogenic vials (Nalgene) and 50 µl of haemolymph from each stored in a separate vial for Haemocyanin analysis. All vials were frozen in liquid Nitrogen and stored in a freezer at −25 °C.

Haemolymph samples were deproteinated as per Paterson and Spanoghe (1997). Proteins were inactivated by adding an equal volume of 0.6 M perchloric acid then separated by centrifugation. The supernatant was neutralized with 3 M potassium hydroxide. Samples were then stored at −25 °C for further analysis. A total of 53 crabs were used for D-Glucose trials and 54 for L-Lactate, comprising of all three size groups.

D-Glucose

D-Glucose concentrations were measured using a D-Glucose assay kit (Sigma GAGO20-1KT) as per Barrento *et al.* (2010). Deproteinated samples were measured spectrophotometrically at 540 nm using a microplate reader (Molecular Devices, Spectramax M5). D-Glucose concentrations (mmol l^{-1}) were calculated from a calibration curve using standards of known concentration.

L-Lactate

L-Lactate concentrations in deproteinated haemolymph samples were measured using L-Lactate reagent (Trinity Biotech; 735–10), per the procedure described by Barrento *et al.* (2010). Samples were transferred to 96-well flat-bottom microplates then measured spectrophotometrically at 540 nm. L-Lactate concentrations (mmol l^{-1}) were calculated from a calibration curve using standards of known concentration (Trinity Biotech, Wicklow, Ireland; L-Lactate standards 735- 11).

Haemocyanin

Haemocyanin concentrations were determined spectrophotometrically. 50 µl of haemolymph was diluted with 2 ml chilled distilled water and 280 µl of the mixture

transferred to a 96-well flat-bottom microplate and absorbances read at 335 nm. Haemocyanin concentrations (mg/ml) were calculated from the molar extinction coefficient $E_{1\ cm}^{mM} = 17.26$ as previously described by Harris and Andrews (2005).

Respiration

Oxygen consumption trials were performed with 15 juvenile (≤79 mm carapace width) crabs. A 46 l flow through tank was set up as a water bath, with filtered, UV sterilised seawater connected to a sump tank and temperature control unit to ensure temperature stability (12 °C ± 0.1 °C). A 0.3 L respiration chamber was filled with UV sterilised filtered seawater and placed into the water bath. Oxygen consumption was recorded using PreSens PSt3 sensor spots (detection limit 15 ppb) and an optical oxygen meter (PreSens Fibox 3). Changes in air saturation (%) in the chamber were measured continuously, with a starting O_2 concentration of 100% saturation, for approximately 30 min or until 60% air saturation was reached. The system was calibrated as described by McLean and Todgham (2015) to obtain hyperoxic conditions and account for bacterial load present in the seawater. Calibration for hypoxic levels was obtained by following the manufacturers guidelines (PreSens GmbH, Regensburg, Germany).

For each trial crabs were randomly selected then sexed, weighed and carapace widths measured. The respiration chamber valve was opened and the crab was placed into the respiration chamber. After an acclimation period of 1 h, the valve was closed and measurements commenced. Percentage air saturation was measured for each crab and converted to oxygen consumption (mg/g/h).

Statistical analysis

Results were expressed as mean value ± standard error (SEM). When data met ANOVA assumptions (according to Shapiro-Wilk test for normality and Levene's test for equality of error variances) multiple comparison tests (2-way ANOVA, repeated measures ANOVA) were conducted to reveal differences between groups. The random factor of trial number was not included during statistical analysis. If data could not meet ANOVA assumptions, non-parametric analysis (Scheirer-Ray-Hare, Friedman test) was

performed. Post-hoc analysis for parametric (LSD test) and non-parametrical (Wilcoxon Signed Rank test) were conducted. All statistics were tested at a probability of 0.05 with the software IBM SPSS Statistics (version 23).

2.3 Results

Behavioural analysis

Activity level

There were no significant differences in the activity levels between male and female crabs in both day (male - 7.8 ± 1.4%, female – 7.6 ± 1.8%) and night conditions (male - 33.9 ± 4.1%, female – 37.3 ± 5%). There was a significant effect on the time of the day ($F(1,157)=99.07$, $p<0.001$, 2-way ANOVA) and size ($F(2,156)=16.55$, $p< 0.001$, 2-way ANOVA) on activity levels (Figure 2.1). Pairwise comparison revealed that during day conditions there was a significant difference in activity level between all size groups ($p < 0.01$, Paired t-test). The activity level of the crabs decreased by size (16.5 ± 2.8% – small, 7.3 ± 1.6% - medium, 3 ± 0.7% - large). During night conditions, there was an overall increase in activity levels, compared to day, with size proving to be a significant factor ($F(1,89)=3.74$, $p<0.05$, 2-way ANOVA) in addition to time ($F(1,90)=99.07$, $p<0.001$, 2-way ANOVA). Post-hoc analysis indicated a significant difference between small and large ($p<0.05$, LSD test) and medium and large ($p<0.05$, LSD test) crabs. Activity level decreased by size from 44.7 ± 6.8% (small) to 43.1 ± 7.7% (medium) and finally 26.9 ± 3.1% (large).

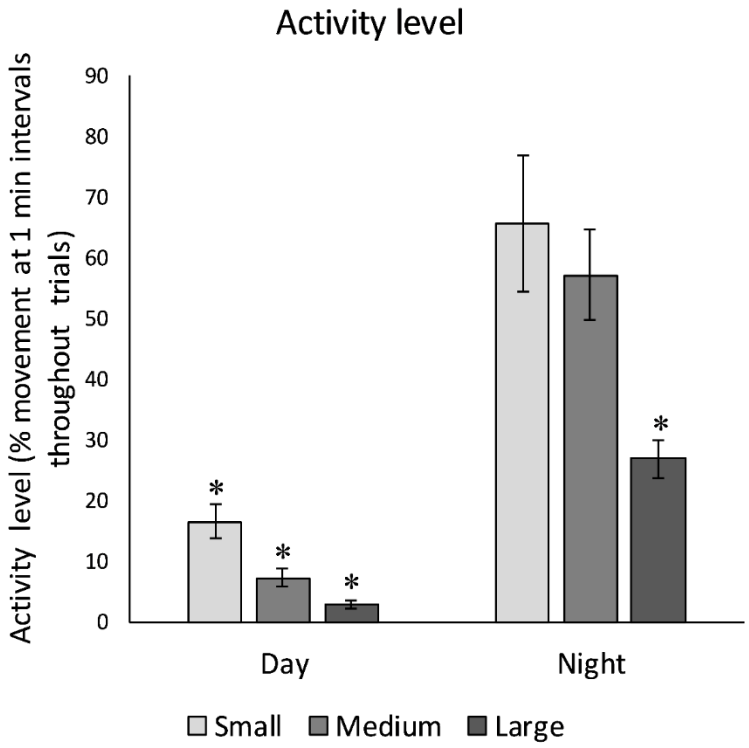


Figure 2.1. Activity level (mean ± SEM), calculated as percentage movement broken down in to 1 min intervals of the total trial duration, for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) in both day and night conditions, along with combined totals. Asterisks represent a significant difference between size groups ($p < 0.05$). $N_{DAY} = 92$, $N_{NIGHT} = 67$.

Flicking rate

There was a significant difference in flicking rate between size groups ($F(2,27)=4.04$, $p<0.05$, Student's t-test), with small crabs having significantly lower flicking rate (10.2 ± 3.9 flicks/min) compared to large (34.7 ± 7.9 flicks/min) crabs (Figure 2.2). There was no significant difference in flicking rate between males (25.9 ± 6.7 flicks/min) and females (19.2 ± 3.8 flicks/min).

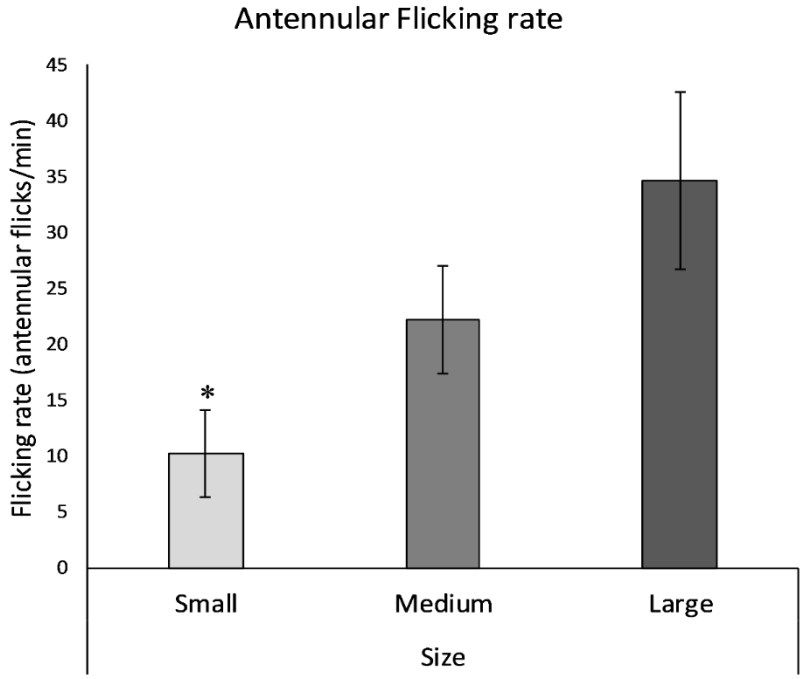


Figure 2.2. Flicking rate (mean ± SEM) calculated as the number of flicks per minute for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) , also split by sex. Small crabs exhibited a significantly lower antennular flicking rate than medium and large crabs with no significant differences found between medium and large individuals. Asterisks represent a significant difference between size groups ($p<0.05$). N=30.

Physiological analysis

Haemolymph

D-Glucose

D-Glucose concentration ranged in small crabs from 0.09 mmol l^{-1} to 0.55 mmol l^{-1}, in medium crabs from 0.18 mmol l^{-1} to 1.10 mmol l^{-1} and in large crabs from 0.05 mmol l^{-1} to 1.40 mmol l^{-1}. Larger crabs had significantly ($F(2,50)=9.54$, $p<0.01$, 2-way ANOVA) higher concentrations of D-Glucose (0.62 ± 0.05 mmol l^{-1}), than the small size group (0.33 ± 0.08 mmol l^{-1}) and D-Glucose levels varied significantly over time ($F(3,16)=7.12$, $p<0.01$, 2-way ANOVA) (Figure 2.3). For medium and large crabs mean D-Glucose levels steadily increased to a peak at 8 h

(medium - 0.68 ± 0.12 mmol l^{-1}, large – 0.92 ± 0.12 mmol l^{-1}) after the 0 h samples (medium – 0.36 ± 0.12 mmol l^{-1}, large – 0.39 ± 0.06 mmol l^{-1}). The final samples taken after 24 h (medium – 0.44 ± 0.10 mmol l^{-1}, large – 0.66 ± 0.11 mmol l^{-1}) showed mean levels closer to baseline values. D-Glucose concentrations for small crabs showed a marginal increase from 0 h to 4 h but remained relatively constant throughout the 24 h sampling period. Female and male crabs' D-Glucose levels did not differ significantly in any of the size groups or at different sampling times.

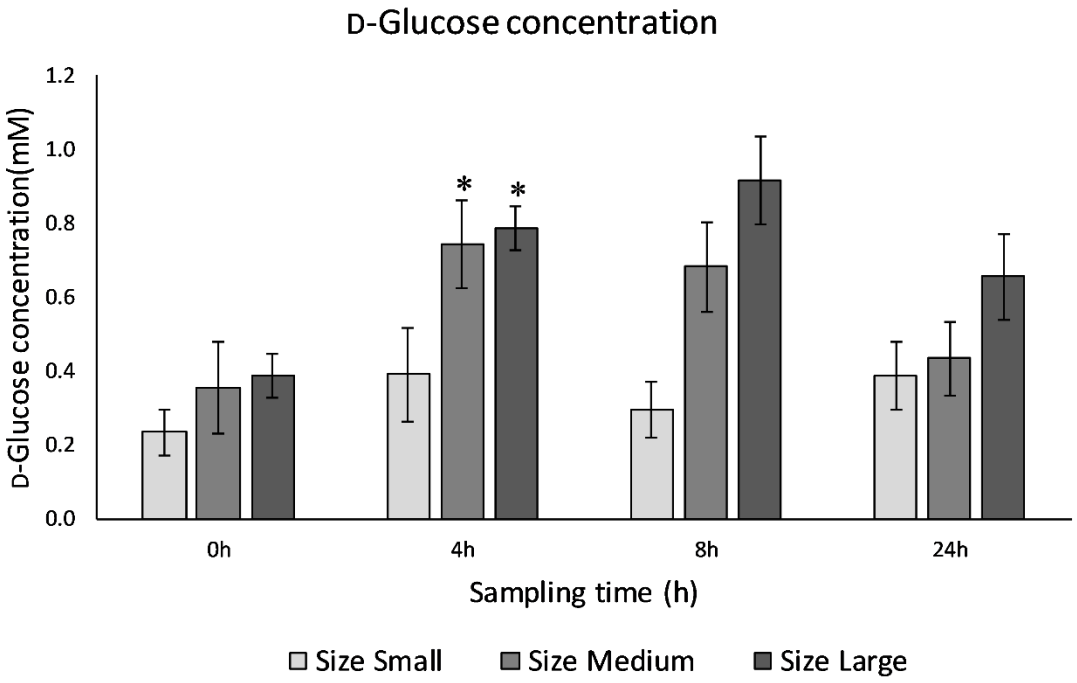


Figure 2.3. D-Glucose concentration (mean ± SEM) over the 24-hour sampling period for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) of crabs. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). Asterisks represent a significant difference between 0 h within the respective size groups ($p<0.05$). N=53.

L-Lactate

L-Lactate concentrations ranged from 0.09 mmol l^{-1} to 7.20 mmol l^{-1} in small crabs, 0.09 mmol l^{-1} to 8.98 mmol l^{-1} in medium crabs, and 0.03 mmol l^{-1} to 12.65 mmol l^{-1} in large crabs. Size had a significant effect on L-Lactate ($p<0.05$, Scheirer-Ray-Hare test) concentration (Figure 2.4). L-Lactate concentration varied significantly over time ($p<0.05$, Scheirer-Ray-Hare test) for the small size group with values obtained at

4 h, 8 h and 24 h all showing significant differences to the 0 h but not from each other. Larger crabs have higher concentrations of L-Lactate (1.98 ± 0.21 mmol l^{-1}), than the small size group (1.47 ± 0.32 mmol l^{-1}). L-Lactate concentrations for medium and large crabs remained relatively constant throughout the initial 4 h (medium – 1.54 ± 0.73 mmol l^{-1}, large – 1.89 ± 0.38 mmol l^{-1}), after a decrease from 0 h (medium – 3.10 ± 0.96 mmol l^{-1}, large – 2.19 ± 0.46 mmol l^{-1}), but showed an increase at the 8-h mark (medium – 1.97 ± 1.07 mmol l^{-1}, large – 3.73 ± 1.29 mmol l^{-1}). In small crabs, there were significant decreases from 0 h (3.34 ± 0.78 mmol l^{-1}) to 4 h (0.44 ± 0.19 mmol l^{-1}). The levels then remained constant including the sample taken at 24 h (0.48 ± 0.13 mmol l^{-1}). L-Lactate concentrations did not significantly vary between sexes.

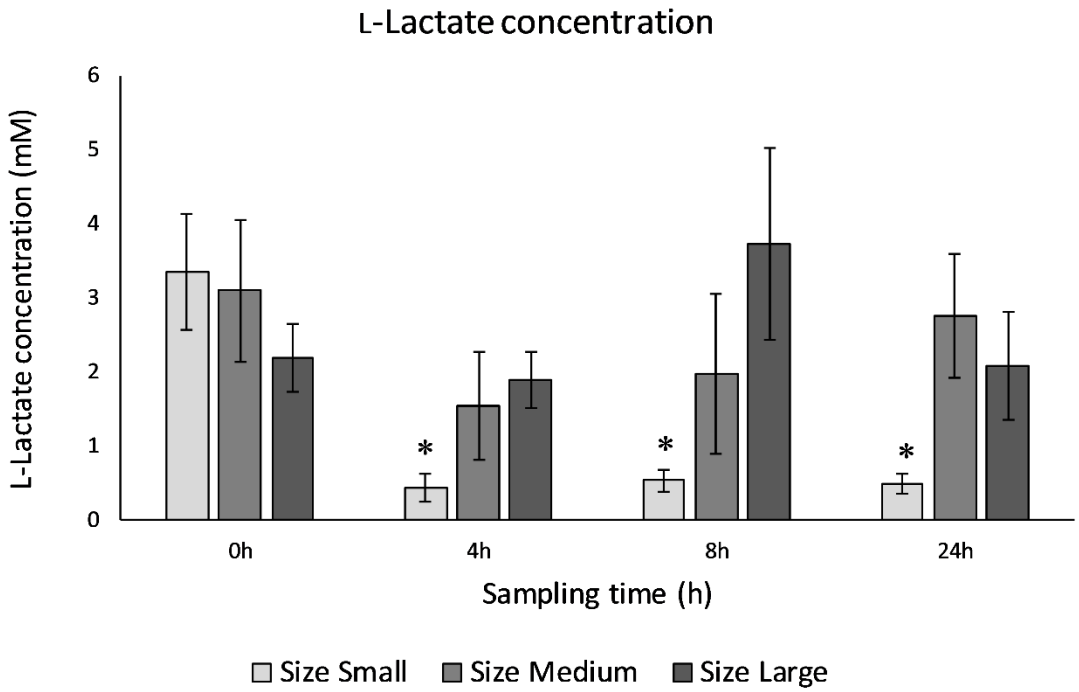


Figure 2.4. L-Lactate concentration (mean ± SEM) over the 24-hour sampling period for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) of crabs. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). Asterisks represent a significant difference between 0 h within the respective size groups ($p < 0.05$). N=54.

Haemocyanin

Haemocyanin concentrations ranged from 10.04 mg/ml to 96.10 mg/ml in small crabs, 23.80 mg/ml to 89.48 mg/ml in medium crabs, and 9.40 mg/ml to 79.48 mg/ml in large crabs. Haemocyanin levels showed individual variability but were not variable over time. Small (52.2 ± 10.2 mg/ml), medium (52 ± 8.6 mg/ml) and large (43.5 ± 2.7 mg/ml) size groups show that the average Haemocyanin concentration does not significantly differ by size. Sex has no effect on the Haemocyanin concentrations with males (49.2 ± 3.5 mg/ml) and females (43.1 ± 4.1 mg/ml) recording similar mean values.

Haemolymph specific gravity

The Specific Gravity (SG) of haemolymph remained relatively constant over time with no significant differences between sample times. SG values ranged from 1.01 to 1.10 in small crabs, 1.01 to 1.12 in medium crabs, and 1.01 to 1.13 in large crabs. Small (SG = 1.058 ± 0.006) and medium (SG = 1.048 ± 0.007) crabs had similar average SG whilst large individuals (SG = 1.073 ± 0.008) had higher average SG but this was not found to be significant. There were no significant differences between average haemolymph SG of male (SG = 1.059 ± 0.006) and female (SG = 1.058 ± 0.006) crabs.

Respiration rate

Respiration rates of juvenile crabs ranged from 0.013 mg O_2/g/h to 0.091 mg O_2/g/h. Respiration rates were significantly different between males and females ($F(1,13)=6.12$, $p<0.05$, Student's t-test) with females (0.06 ± 0.008 mg O_2/g/h) consuming more oxygen than males (0.04 ± 0.006 mg O_2/g/h).

2.4 Discussion

The increased activity level found in small crabs, compared to larger bodied individuals, could aid the crab in survival by actively seeking a secure shelter area to avoid predators. Shelter utilisation is common in many decapod crustaceans (Chapman and Rice, 1971; Hockett and Kritzler, 1972; Hazlett and Rittschof, 1975; Hill, 1976) and provides refuge from predation. *Cancer pagurus* has been shown to inhabit pits in the sand when inactive (Hall *et al.,* 1991) and were observed utilising rocks, crevices and kelp holdfasts during sample collection for this experiment. Small crabs inhabiting the

sub- littoral zone would be subjected to increased predation, environmental factors (freshwater influx, variable temperatures and salinities) and competition for shelter. Larger crabs that tend to inhabit deeper areas offshore and are likely to experience a decrease in predation rate due to individuals reaching a size where the ability of a single predator to consume them is surpassed (Paine, 1976). Kelp forests and algal beds have significantly higher biomass productivity than the open ocean (Mann, 1973; Ricklefs and Miller, 2000; Park, 2001) which may result in less competition for shelter. Both a decrease in predation and com- petition for resources may result in lower overall activity levels. The lower activity levels in larger mature crabs were confirmed when a decrease in overall activity of approximately 13% between small juveniles and mature adults during the day and around an 18% decrease during the night were observed. Previous studies have highlighted the nocturnal behaviour of *C. pagurus* adults (Skajaa *et al.,* 1998), however this is the first confirmation, to our knowledge, that juveniles follow similar patterns. Active foraging takes place at night (Seed, 1969; Skajaa *et al.,* 1998), with peaks at dawn and dusk, for all size groups. This nocturnal foraging behaviour was confirmed by an increase in activity level of at least 24% approx. at night across all size groups. Whilst ad-hoc observations in holding tanks during this experiment showed that individuals will consume food during the day with an increase in active foraging and consumption during dusk through until dawn. This nocturnal activity may be in response to visually receptive predators that commonly prey on *C. pagurus* such as birds, cod, seals and wolffish (Rae, 1967, 1968; Rae and Shelton, 1982; Skajaa *et al.,* 1998). Nocturnal foraging may also widen the food spectrum of *Cancer pagurus* and result in increased pursuit success as has been found in *Carcinus maenas* (Naylor, 1960). Sex did not have a significant effect on the activity levels despite previous studies suggesting that male crab movement is limited whilst females migrate over large distances relatively rapidly (Edwards, 1979; Brown and Bennett, 1980). Whilst smaller crabs tend to have higher activity levels, larger mature crabs may rely more on chemical sensing through antennular flicking and less on physical exploration during day conditions. In work conducted by Woodruff *et al.* (2013) on the closely related Dungeness crab, *Metacarcinus magister*, the number of antennular flicks per minute ranged from 5.6 to 40 which coincided with earlier work undertaken by Pearson *et al.* (1979), and are in line with the results obtained in this experiment of 5 to 43flicks/min. There were no significant differences between flicking rates of male and

female crabs of any size. Previous studies found that male helmet crabs, *Telmessus cheiragonus*, can detect pheromones, released through the female's urine, via their first antennae (Kamio *et al.,* 2000). If pheromones are utilized by *C. pagurus* females in mate attraction, then there may be seasonal variations whereby males will have increased antennular flicking.

D-Glucose and L-Lactate cycles are linked and followed a natural circadian rhythm with a rise in D-Glucose, and a subsequent fall in L-Lactate concentrations throughout the day. In crustaceans both components have been shown to be affected by environmental stressors (Kallen *et al.,* 1990; Reddy *et al.,* 1996; Chang *et al.,* 1998). Both D-Glucose and L-Lactate values obtained corresponded well with those found in previous literature (Watt *et al.,* 1999; Lorenzon *et al.,* 2008; Barrento *et al.,* 2010; Barrento *et al.,* 2011). D-Glucose, which is the primary fuel for ATP formation in crustaceans, is essential to maintain metabolic processes (Barrento *et al.,* 2010). D-Glucose levels continually rise in relation to increased locomotor activity (Hamann, 1974; Reddy *et al.,* 1981; Kallen *et al.,* 1988; Kallen *et al.,* 1990; Tilden *et al.,* 2001). This suggests that levels would continue to rise throughout the night, before decreasing back to original levels at the next sampling time exactly 24 h later. Activity in crabs should partially be reflected in D-Glucose concentrations (Briffa and Elwood, 2001). A negative correlation between D-Glucose levels and vigour has been shown where healthy individuals have lower levels and weak and moribund crabs have become hyperglycaemic (Barrento *et al.,* 2010). D-Glucose levels have been found to vary significantly in individual crabs as it is controlled by individual physiological status and reactions to external stimulus (Matsumasa and Murai, 2005).

L-Lactate, as a metabolite, is typically an indicator of anaerobic respiration due to impaired gill function or hypoxic/anoxic conditions (Durand *et al.,* 2000). L-Lactate levels were found to be highly variable in individuals of all sizes. As previously reported by Barrento *et al.* (2010) there were no differences in haemolymph components between males and females. Haemocyanin levels have been shown to increase during periods of hypoxia, where additional proteins are required to transport oxygen (Hagerman *et al.,* 1990). Haemocyanin concentrations are not size dependent and do not follow the same circadian rhythm as L-Lactate and D-Glucose, however it was shown in *Carcinus maenas* that there may be an oxygen debt that has to be repaid during re-

immersion, such as high tide, which may affect Haemocyanin on a tidal-linked cycle (Simonik and Henry, 2014). Low oxygen availability may be somewhat counteracted by the high degree of respiratory independence that has been shown to occur within this species (Bradford and Taylor, 1982). Bottoms (1977) and Burnett and Bridges (1981) described that short-term rhythmic patterns are present in the respiration of this species, where there are alternating periods of apnoea and bradycardia in the branchial chambers. It has been suggested that these apnoea pauses could enable the animal to save on metabolic energy during periods of inactivity by reducing the energy spent on pumping both water and blood (Bottoms, 1977; McMahon and Wilkens, 1977; Burnett and Bridges, 1981). Respiration rates could therefore vary quite significantly between periods of apnoea (pausing behaviour) and normal pre-pause respiration. Respiration rates of mature *C. pagurus* were reported as being 28.03 mg O_2/g/h during pre-pause and 4.42 mg O_2/ g/h post-pause (Bradford and Taylor, 1982). In a study by Burnett and Bridges (1981) it was found that the crabs were utilising this pausing behaviour 40–50% of the time. The respiration rates of juvenile crabs observed in this experiment correspond well with those of different crab species, of similar size, in previous studies: velvet swimming crab, *Necora puber*, (0.21 ± 0.119 mg O_2/g/h (Small *et al.,* 2010)); spider crab, *Hyas araneus*, (0.025 mg O_2/g/h (Camus *et al.,* 2002)); and Dungeness crab, *Cancer magister*, (0.044 mg O_2/g/h (Johansen *et al.*, 1970)). Respiration values found in the literature for the shore crab, *Carcinus maenas*, vary from 0.036 mg–0.126 mg O_2/g/h (Newell *et al.*, 1972; Taylor and Butler, 1978; Taylor and Wheatly, 1979).

There is a natural cycle of L-Lactate and D-Glucose concentrations in the haemolymph of *Cancer pagurus*, which must be fully understood to successfully measure the effects of anthropogenic and environmental stressors on this species. Both cycles appear to approximately follow a 24-hour period that is directly linked with activity level and nocturnal behaviour. There is a clear size-linked difference in L-Lactate and D-Glucose concentrations in crabs, with high individual variability. Haemocyanin and the SG of the haemolymph is not size dependant and do not follow a 24-hour cycle. The activity levels of this species increase significantly during the night, with peaks at dawn and dusk, when foraging takes place. Respiration rate varies between male and female crabs, with female crabs consuming more oxygen per hour. The use of L-Lactate and D-Glucose concentrations, as a stress component, in future research must factor in the

natural diel cycles and individual variation to accurately determine potential affecting factors. The sole use of adult crabs in previous studies does not necessarily best represent the potential effects on this species.

Chapter 3. Baseline measurements of behavioural and physiological stress markers in the commercially important decapod European lobster, *Homarus gammarus* (L.)

3.1 Introduction

The marine environment is subjected to a range of anthropogenic stressors, including, but not limited to: noise (Markus & Sánchez, 2018), pollutants (Doney, 2010), acidification (Fabry *et al.*, 2008), plastic waste (Vegter *et al.*, 2014), and electromagnetic fields (Scott *et al*., 2018a). Stressors may not necessarily be independent of one another, but rather lead to a multi-stressor scenario which could result in unpredictable effects on the environment (Chapman, 2017). Such potentially negative effects are likely to ultimately impact marine organisms, leading to stress responses (e.g. Chang, 2005; Scott *et al*., 2018a). To understand the effects of such stressors on marine organisms, there is a need to first understand the natural state of individual species in stress limited conditions. Information on this is currently lacking for the European lobster (*Homarus gammarus*).

H. gammarus is a commercially important decapod crustacean with a distribution ranging from Norway to Morocco (Triantafyllidis *et al.*, 2005; Moland *et al.*, 2011), being found in coastal areas to depths of up to 50 m and occasionally as deep as 150 m (Holthuis, 1991), across a temperature range of 0–25 °C (Qadri *et al.*, 2007), which coincides with their distributional range. In the United Kingdom in 2016, *H. gammarus* accounted for 3,300 tonnes of landed shellfish valued at £39.5 million (Marine Management Organisation, 2017), with £14 million of this being from Scottish based vessels (Scottish Government, 2017).

H. gammarus are generally considered to be nocturnal (Moland *et al.*, 2011), inhabiting rocky outcrops and sandy expanses during the day (Smith *et al.*, 2001; Mehrtens *et al*., 2005; Galparsoro *et al.*, 2009), with increased activity after sunset (Smith *et al*., 1999; Moland *et al.*, 2011) cued by a photoperiod circadian rhythm (Smith *et al*., 1998, 1999; Jury *et al*., 2005). This diel activity pattern benefits their energetic efficiency regarding foraging and reduces susceptibility to predation during the day (Smith *et al*., 1998; Moland *et al.*, 2011). A study by Mehrtens *et al*. (2005) on juvenile *H. gammarus* revealed

a negative correlation between lobster size and locomotory activity. Activity levels are a useful stress measure, as decapods have shown an avoidance response to stressors in the marine environment (e.g. Filiciotto *et al.,* 2014; Smyth *et al*., 2014). A study by Smyth *et al*. (2014) highlighted avoidance behaviour in *H. gammarus* and edible crab (*Cancer pagurus*) when exposed to an altered salinity environment and Celi *et al*. (2013) showed that freshwater red swamp crayfish (*Procambarus clarkii*) alter their aggressive behaviour patterns dependent on the presence of acoustic stimuli.

Analysis of haemolymph is a common method for measuring health and stress in crustaceans (e.g. Hagerman, 1986; Lorenzon *et al*., 2011; Scott *et al*., 2018a; Ooi *et al.,* 2019). Haemolymph protein concentration has been used to assess *H. americanus* for nutritional quality via refractometry (Leavitt and Bayer, 1977; Ozbay and Riley, 2002). Protein concentration within crustacean haemolymph is proportional to the haemolymph's refractive index (Smith and Dall, 1982; Moore *et al*., 2000; Lorenzon *et al*., 2011; Scott *et al*., 2018a, 2018b). Spectrophotometric assessment of haemolymph can also be applied to analyse concentration of proteins such as Haemocyanin (Zeis *et al.,* 1992; Scott *et al*., 2018a, 2018b), which has been shown to correspond with refractometry results (Spoek, 1974; Hagerman *et al.,* 1990; Lorenzon *et al*., 2011). Haemocyanin, the primary oxygen carrying protein in crustaceans, has been shown to increase during periods of hypoxia (Hagerman *et al.,* 1990) and has been used in previous experiments to assess stress levels (Hagerman, 1983, 1986).

Total Haemocyte Counts (THCs) are also used as a measure of stress (Durliat and Vranckx, 1983; Persson *et al*., 1987; Smith *et al.,* 1995; Jussila *et al.,* 1997; Lorenzon *et al.,* 2007). A study involving the pink shrimp (*Farfantepenaeus paulensis*) showed that animals in lower salinity expressed a 40% lower THC than the controls (Perazzolo *et al.,* 2002).

D-Glucose concentrations have been shown to rise continually in relation to increased locomotor activity (Hamann, 1974; Reddy *et al.,* 1981; Kallen *et al*., 1988; Kallen *et al*., 1990; Tilden *et al.,* 2001), as well as in response to stress such as emersion (Morris *et al*., 1986; Taylor *et al.,* 1997; Bergmann *et al*., 2001; Speed *et al.,* 2001; Lorenzon *et al.,* 2007), pollutants (Nagabhushanam and Kulkarni, 1981; Machale *et al.,* 1989; Reddy and Bhagyalakshmi, 1994; Lorenzon *et al*., 2000), and disease (Lorenzon *et al*., 1997). D-Glucose is the primary fuel for Adenosine Triphosphate (ATP) production in crustaceans

and is essential in maintaining metabolic processes (Verri *et al.*, 2001; Jimenez and Kinsey, 2015).

L-Lactate concentrations in crustacean haemolymph has also been shown to increase during exposure to various stressors (Spicer *et al.*, 1990; Taylor *et al.*, 1997; Durand *et al.*, 2000; Bergmann *et al*., 2001; Ocampo *et al.*, 2003; Lorenzon *et al.*, 2007; Scott *et al*., 2018a). Increasing L-Lactate concentrations have been found to increase Hemocyanin oxygen affinity in crustaceans (Bridges and Morris, 1986; Zeis *et al.*, 1992).

The aim of the present chapter is to determine the natural behavioural and physiological baseline characteristics of minimally stressed *H. gammarus* using common previously utilised stress markers, including: activity level, refractometry of haemolymph (Scott *et al*., 2018a), Haemocyanin concentration (Spoek, 1974; Hagerman *et al.*, 1990), THC (Durliat and Vranckx, 1983; Persson *et al*., 1987; Smith *et al.*, 1995; Jussila *et al.*, 1997), D-Glucose and L-Lactate haemolymph concentrations (Taylor *et al.*, 1997; Durand *et al.*, 2000; Bergmann *et al*., 2001; Scott *et al*., 2018a, 2018b).

3.2 Materials and Methods

Intermoult lobsters were collected from local fishermen within the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK), stored in dark boxes with a seawater supply and transported back to the research facility within 4 h. Lobsters were sexed, carapace length measured (mm), weighed (g), and assigned a condition index adapted from Scott *et al.* (2018a) to ensure lobsters were classed as 'good' or 'perfect', to ensure minimal external influence through lack of vigour, before use in experimentation. Size categories were determined using the Minimum Landing Size (MLS) of 88 mm as a reference (undersize < 88 mm and legal size ≥ 88 mm). These sizes correspond to the average size at maturity of *H. gammarus* in Scotland as per Lizarraga *et al.* (2003). An approximately equal split of males and females was used throughout the experiments. Lobsters were kept in individual 500 L tanks, which were connected to a flow-through system at ambient sea temperature (9–10 °C) and a natural photoperiod, via a transparent roof on the research aquarium for an acclimation period of one week. Lobsters were fed on cooked, and live blue mussels (*Mytilus edulis*), except 24 h prior to and during experimentation. Experimentation was conducted in November 2018.

Behavioural analysis

Activity level

Six 70 L tanks were connected to three 800 L temperature-controlled sump tanks which received a constant supply of seawater. Visual shading provided by plastic panels was placed down the sides of each tank to prevent visual disturbance. Mesh with a wide aperture was clamped on top of the tanks to prevent lobsters from escaping during the trial period. Seawater was pumped using a submersible pump and Hozelock adjustable control panel at a rate of around 3 L min^{-1} $tank^{-1}$. Tank parameters such as salinity (ppt), temperature (°C), and dissolved oxygen (DO%) were monitored throughout experimentation and remained constant. An acclimation period of 1 h was given to each lobster in their respective tanks prior to experimentation. After each trial, all tanks and sump tanks were drained, sterilised (Virkon aquatic), and refilled. Six waterproof InfraRed (IR) cameras (Sannce 1080p IR surveillance DVR system) were suspended above the experimental tanks and set to record continuously throughout each 24 h trial. Hour-long segments of each video file were separated into still images at 1-min intervals and analysed using Solomon Coder (beta version 17.03.22). Lobsters were considered to have moved when they were in a different location when compared to the previous still image.

During the study period (8–11 November 2018) sunrise varied from 07:30 to 07:36 and sunset from 16:14 to 16:08. For activity level analysis the 24-h cycle was broken down into four time periods: first half of the day (07:00–12:00), second half of the day (12:00–17:00), first half of the night (17:00–00:00), and second half of the night (00:00–07:00). The number of movement occurrences during each time period was divided by the number of hours in that time period to give activity levels (movement/hour). A total of 15 individuals were analysed (undersize $N = 6$ and legal size $N = 9$).

Physiological analysis

Haemolymph analysis

During the 24-h haemolymph collection period, lobsters < 100 mm were kept individually in the 30 L acclimation tanks, with the black boxes acting as temperature controlled (TECO TK2000) water baths. Lobsters > 100 mm were kept individually in 50 L with black ABS boxes used, as with the smaller lobsters, as temperature controlled water baths.

Haemolymph was sampled throughout 24 h at 09:00 (0 h), 15:00 (6 h), 21:00 (12 h), and 09:00 (24 h). Sampling times were chosen to correspond closely to observed changes throughout the natural circadian rhythm found in *H. americanus* (Golet *et al.*, 2006) and *C. pagurus* (Scott *et al*., 2018a, 2018b). Sampling per lobster did not exceed 60 sec to minimise handling stress.

Haemolymph was extracted from the base of the fifth walking leg, after cleaning the membrane with 70% ethanol, using 1 ml syringes with 25 G sterile needles stored previously at –25 °C to prevent coagulation of the haemolymph once extracted. A maximum 300 µl of haemolymph was collected from undersize lobsters and 700 µl from legal size lobsters. Haemolymph samples were divided for analysis as follows: 50 µl refractometry, 50 µl Haemocyanin, 50 µl THC, 300 µl (50 µl undersize lobsters) D-Glucose, and 300 µl (50 µl undersize lobsters) L-Lactate. Haemolymph was placed in 1.5 ml cryogenic vials (Nalgene), which were stored on ice for subsequent THC and Haemocyanin analysis or immediately deproteinised and stored at –25 °C till further analysis. A total of 32 individuals were analysed (undersize $N = 13$, legal size $N = 19$) over the 24-h haemolymph trials.

Haemolymph density and Haemocyanin concentration

Density of the haemolymph was measured using a density-salinity refractometer (TMC V^2 refractometer) with automatic temperature compensation. The refractometer range was between 1,000–1,070, with 1 g l^{-1} accuracy. A dilution of 1:1 (V/V) was performed with distilled water if values exceeded the upper limit of the refractometer.

To measure Haemocyanin concentration, haemolymph (50 µl) was immediately diluted with 950 µl of distilled water. Samples were then analysed spectrophotometrically at an

absorbance of 335 nm. Haemocyanin concentrations (mg ml^{-1}) were calculated from the molar coefficient $E_{1\ cm}^{\ mM}$ = 17.26, as previously described by Harris and Andrews (2005).

Total Haemocyte Count (THC)

The collected haemolymph sample (50 µl) was immediately dispensed to a centrifuge vial, containing 150 µl ice cold 5% (v/v) Formaldehyde (Brunel Microscope Ltd) and mixed thoroughly then stored on ice prior to analysis to prevent coagulation (Cornick and Stewart, 1978). Well-mixed samples were then analysed using a Neubauer haemocytometer under magnification of × 100 with a Leica (MC170 HD) compound microscope. To improve accuracy three subsamples were taken from each sample and counted three times. Total Haemocyte Count (THC) was reported as × 10^6 cells ml^{-1}.

D-Glucose

Haemolymph samples were deproteinated prior to D-Glucose and L-Lactate analysis as per Paterson and Spanoghe (1997). Briefly, 600 µl of 0.6 M perchloric acid (BDH 10175) was mixed with the 600 µl haemolymph then centrifuged (Eppendorf 5417C, rotor 30 × 1.5–2ml) at 25,000 g for ten minutes to separate the inactive proteins. The resultant supernatant was mixed with 50 µl of potassium hydroxide (KOH) (BDH 29628), then centrifuged again. 500 µl of the final supernatant was aliquoted to a new vial then stored at –25 °C.

D-Glucose concentrations were analysed using a colorimetric assay kit (Sigma-Aldrich, GAGO20-1KT) as described by Barrento *et al*. (2010). Haemolymph samples were thawed then a 150 µl sub-sample was mixed with 300 µl reagent assay. This mix was then placed in a water bath at 37 °C for 30 min before stopping the reaction using 300 µl of 12 N sulphuric acid (BDH). The mixture was then transferred to a microcuvette (BRAND UV micro-cuvette [BR759200-100EA]) and measured spectrophotometrically at an absorbance of 540 nm. Samples were measured in duplicates to improve accuracy. D-Glucose concentrations were then calculated using a calibration curve of standards with a known concentration (Sigma-Aldrich G3285).

ʟ-Lactate

ʟ-Lactate concentrations were measured using a colorimetric ʟ-Lactate assay kit (Abcam ab65331). 50 µl of reaction mix (46 µl lactate assay buffer, 2 µl lactate substrate mix, and 2 µl lactate enzyme mix), was added to a 50 µl deproteinated haemolymph sample. The reaction and sample mix were then incubated at room temperature (23 °C) for 30 min, then analysed spectrophotometrically at 450 nm twice. The concentrations were determined using a calibration curve of standards with known concentrations (ABCAM, AB65330).

Statistical analysis

Results are expressed as mean ± Standard Error (SE). When the data met parametric assumptions (Shapiro-Wilk test for normality, Levene's test for equality of error variances, Mauchly's sphericity test) multiple comparison tests (pairwise t-test, repeated measures ANOVA, repeated measures ANOVA with multivariate approach) were conducted to highlight any differences between groups. When the data could not meet the parametric assumptions, non-parametric tests (Wilcoxon test, Sign test and Kruskal Wallis ANOVA) were conducted. Post-hoc analysis for parametric data (Tukey's test, pair wise t-test with Holm adjustment) and non-parametric (pairwise Mann-Whitney) were performed. All statistics were tested at a probability of 0.05 using StatSoft Inc. (2005) STATISTICA (data analysis software system) version 7.1 (www.statsoft.com) or R 3.5.0 (activity data) (R Core Team, 2018).

3.3 Results

Behavioural analysis

Activity

Lobster activity peaked within the first half of the night (17:00–00:00) (Figure 3.1.), with the highest peak at 17:00 (Figure 3.2.). There was a significant difference in activity across time periods ($F(3,54)=14.99$, $p<0.05$, repeated measures ANOVA), but no difference in activity across lobster size categories or sex, and no interaction between time period, size category, or sex. Post-hoc analysis (pair wise t-test with Holm adjustment) revealed that all time period comparisons were statistically significant

(p<0.05), except the second half of the day (12:00–17:00) compared to the second half of the night (00:00–07:00).

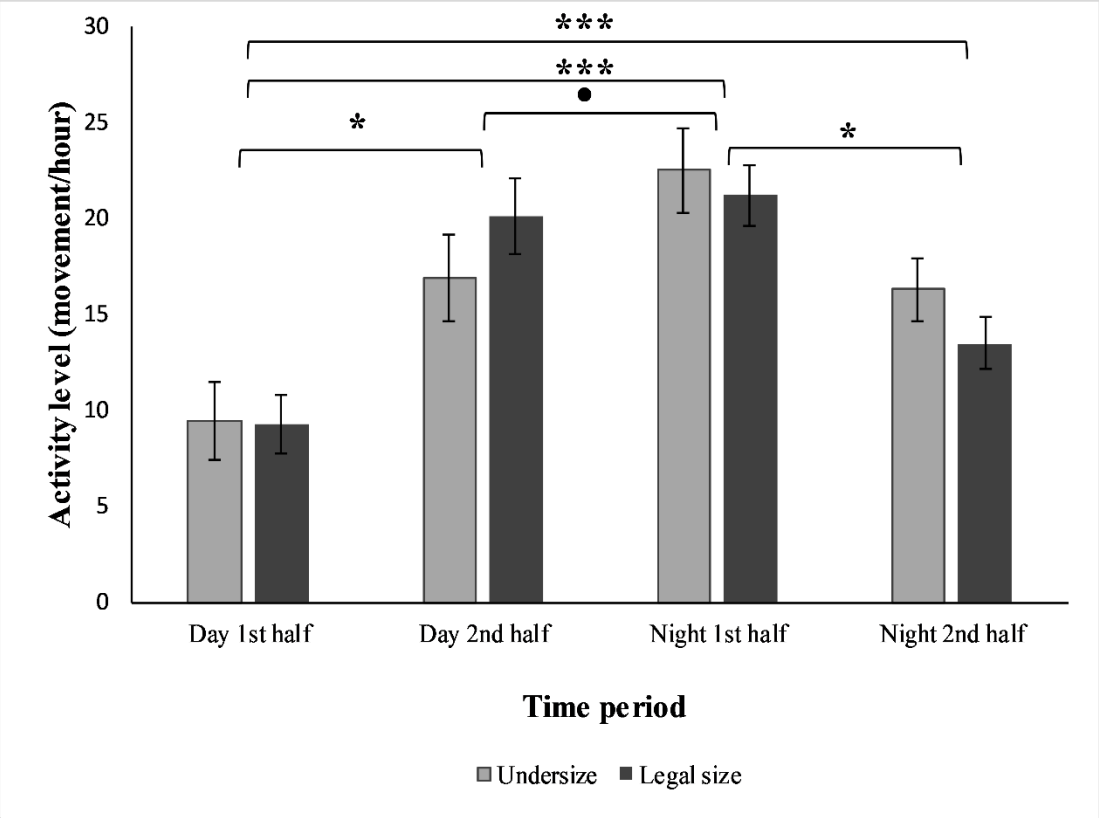


Figure 3.1. Activity level within daily time periods for undersize (< 88 mm) and legal size (≥ 88 mm) *Homarus gammarus* over a 24-hour period. Significance levels between time periods, with sizes combined, from pairwise t-test with Holm adjustment shown as *** $p < 0.001$, * $p = 0.01$, • $0.01 < p < 0.05$. Undersize N=6, legal size N=9.

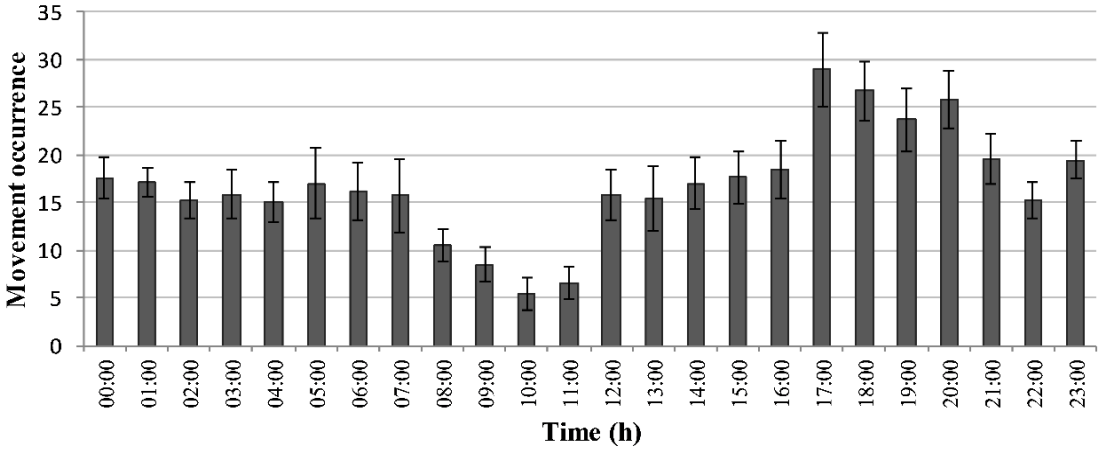


Figure 3.2. Mean number of movement occurrences each hour for *Homarus gammarus* over a 24-hour period in November (combined size and sex). Peak activity levels occurring at dawn (04:00 – 07:00) and dusk (17:00 – 19:00) confirming crepuscular behaviour. N=15.

Physiological analysis

Haemolymph analysis

A summary of *H. gammarus* haemolymph analysis results are shown in Table 3.1.

Table 3.1. Summary of *Homarus gammarus* haemolymph results, stating mean, minimum, and maximum values obtained throughout the study. Note high individual variability of D-Glucose and L-Lactate haemolymph concentrations. Size categories and sex combined with size ranging from 46mm – 148mm carapace length. N=34.

Haemolymph Measurement	Unit	Minimum	Maximum	Mean	Standard Error (SE)
Haemolymph density	g l^{-1}	1014	1116	1073.6	2.2
Haemocyanin concentration	mg ml^{-1}	15.9	123.7	66.7	3.1
Total Haemocyte Count (THC)	× 10^6 ml^{-1}	8.61	121.82	52.74	2.16
D-Glucose	mmol l^{-1}	0.031	1.542	0.458	0.024
L-Lactate	mmol l^{-1}	0.005	0.347	0.075	0.006

Haemolymph density and Haemocyanin concentration

Haemolymph density was significantly higher ($F(1,32)=7.3$, $p<0.05$, repeated measures ANOVA multivariate approach) in male lobsters (1056–1116 g l^{-1}, 1085.22 ± 4.2 g l^{-1}) compared with female lobsters (1014–1092 g l^{-1}, 1067.5 ± 4.5 g l^{-1}). There was no significant difference between undersize (1075.19 ± 4.5 g l^{-1}) and legal-size lobsters (1076.54 ± 4.2 g l^{-1}). Haemolymph density did not change significantly during the 24h sampling period in any of the size groups.

Haemolymph Haemocyanin levels showed a slight elevation after 12 h at 21:00 (81.45 ± 8.2 mg ml^{-1}); however, the difference was not statistically significant. There were no differences between undersize and legal-size lobster haemolymph Haemocyanin concentrations at any sampling point. Haemocyanin concentration in male lobsters was slightly higher (80 ± 6.7 mg ml^{-1}) compared to female lobsters (60.17 ± 7.2 mg ml^{-1}), but it did not prove to be statistically significant.

Total Haemocyte Count

Undersize lobsters showed an increase in THC after 12 h at 21:00, compared to the first sampling time at 09:00 and the second sampling time at 15:00 ($Z=2.48$, $p<0.05$, Wilcoxon signed rank test), while legal size group THC did not change significantly throughout the 24 h sampling period (Figure 3.3.). THC of female lobsters was slightly higher (61.2 ± 5.2 × 10^6 ml^{-1}) than male lobsters (47.33 ± 4.1 × 10^6 ml^{-1}), but did not differ significantly from each other at any sampling point. THC did not differ significantly between undersized (8.6–121.8 × 10^6 ml^{-1}, 54.19 ± 5.1 × 10^6 ml^{-1}) and legal size lobsters (9.2–110.6 × 10^6 ml^{-1}, 54.33 ± 4.2 × 10^6 ml^{-1}).

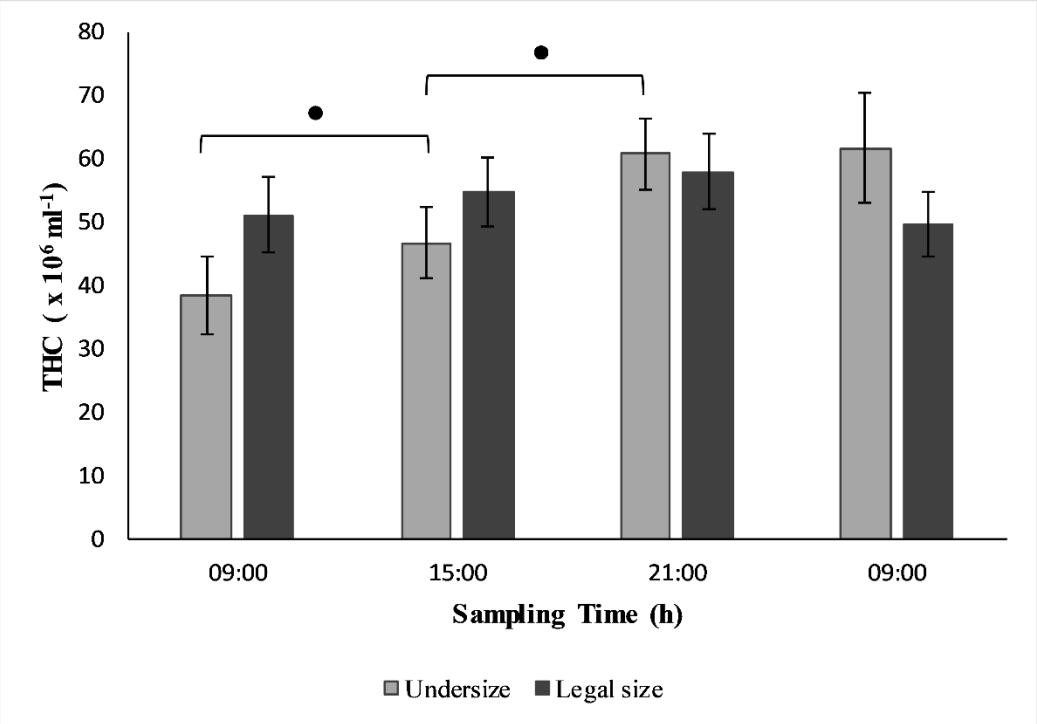


Figure 3.3. Total Haemocyte Count (THC) of undersize (< 88 mm) and legal size (≥ 88 mm) *Homarus gammarus* over a 24-h period. Significance levels between sampling times from Wilcoxon signed rank test shown as • $p < 0.05$. Undersize N=13, legal size N=19.

D-Glucose

Legal size lobster D-Glucose concentrations showed a significant elevation over time ($F(3,30)=5.62$, $p<0.01$, repeated measures ANOVA multivariate approach), with concentrations increasing significantly at all sampled time points after the initial 0 h sampling at 09:00 (0 h/09:00–6 h/15:00 $t(18)=2.7$, $p<0.05$, 0 h/09:00–12 h/21:00 $t(18)=3.49$, $p<0.01$, 0/09:00–24/09:00 h, $t(18)=2.91$, $p<0.01$, paired samples test,) (Figure 3.4.). D-Glucose concentration in undersized lobsters increased to its highest value 0.501 ± 0.04 mmol l^{-1} at 15:00, but this was not significant compared to other sampling times.

D-Glucose concentrations at different sampling times between size groups showed the biggest difference at 21:00 with 0.401 ± 0.06 mmol l^{-1} in undersize lobsters and 0.518 ± 0.06 mmol l^{-1} (mean ± SE) in legal size lobsters; however, it was not significantly different.

There were no significant differences in D-Glucose levels between male and female lobsters in both size groups at any sampling point. Female lobsters showed an increase after 24 h (0.626 ± 0.09 mmol l^{-1}, mean ± SE) compared to male lobsters (0.449 ± 0.06 mmol l^{-1}, mean ± SE); however, this difference was not significant.

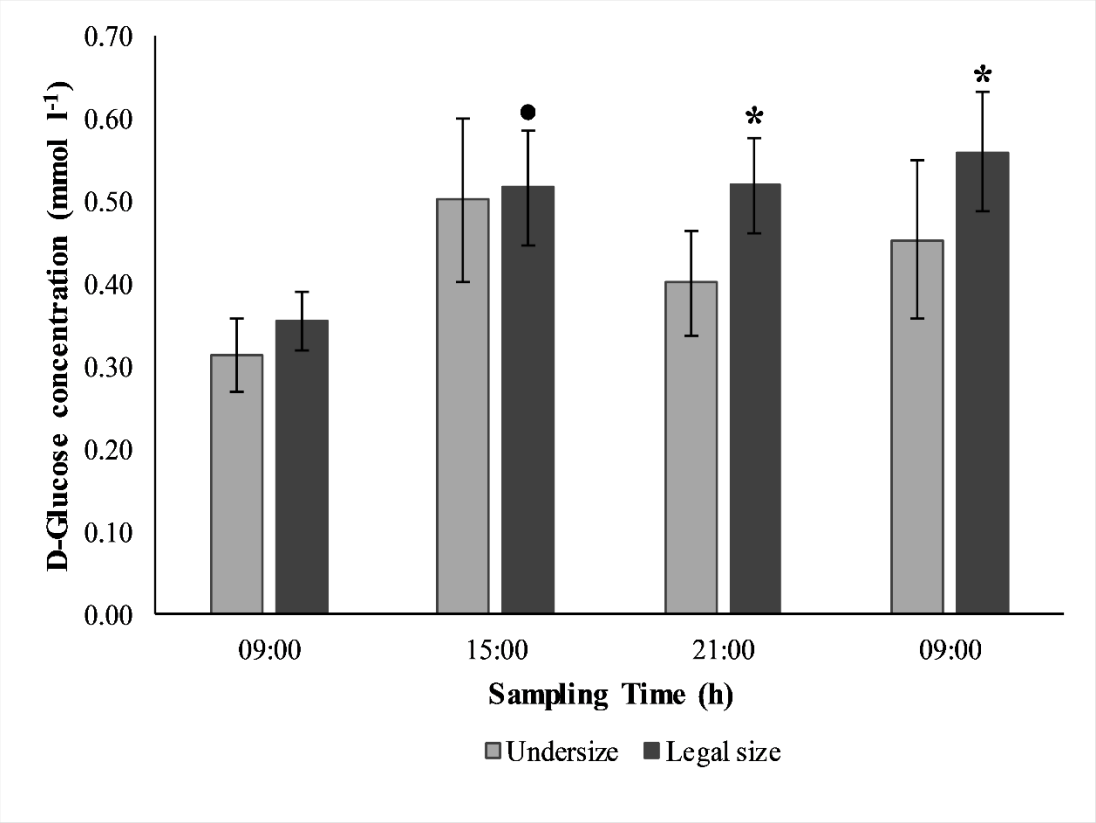


Figure 3.4. Haemolymph D-Glucose concentrations of undersize (< 88 mm) and legal size (≥ 88 mm) *Homarus gammarus* over a 24-hour period. Significance levels between sampling times from paired samples test shown as * $p < 0.01$, • $p < 0.05$. Undersize N=13, legal size N=19.

L-Lactate

L-Lactate concentration varied across lobster size categories (Figure 3.5.). Undersize lobster L-Lactate concentrations showed an increase at 15:00 ($Z=2.1$, $p<0.05$, Wilcoxon signed ranks test) then the lowest concentration at 21:00, with this decrease being statistically significant compared to 09:00 (0 h), 15:00 (6 h), and 09:00 (24 h) ($Z=2.1$, 2.31, 2.49, $p<0.05$, Wilcoxon signed ranks test). Legal size lobsters showed a different

daily pattern with no significant difference in L-Lactate levels during the first 12 h, followed by a significant increase after 24 h (09:00) compared to 0 h (09:00) ($Z=2.84$, $p<0.01$, Wilcoxon signed ranks test,) and 12 h (21:00) ($Z=2.27$, $p<0.05$, Wilcoxon signed ranks test).

Comparing L-Lactate levels between the two size groups at the different sampling times, undersize lobsters had significantly higher L-Lactate concentrations at 0 h (09:00) and 6 h (15:00) compared to legal size lobsters ($H(1,26)=5.33$, $p<0.05$, $H(1,26)=7.14$, $p<0.01$, Kruskal-Wallis test).

When sexes were compared there were no significant differences in L-Lactate levels between male and female lobsters in any size groups at any sampling point.

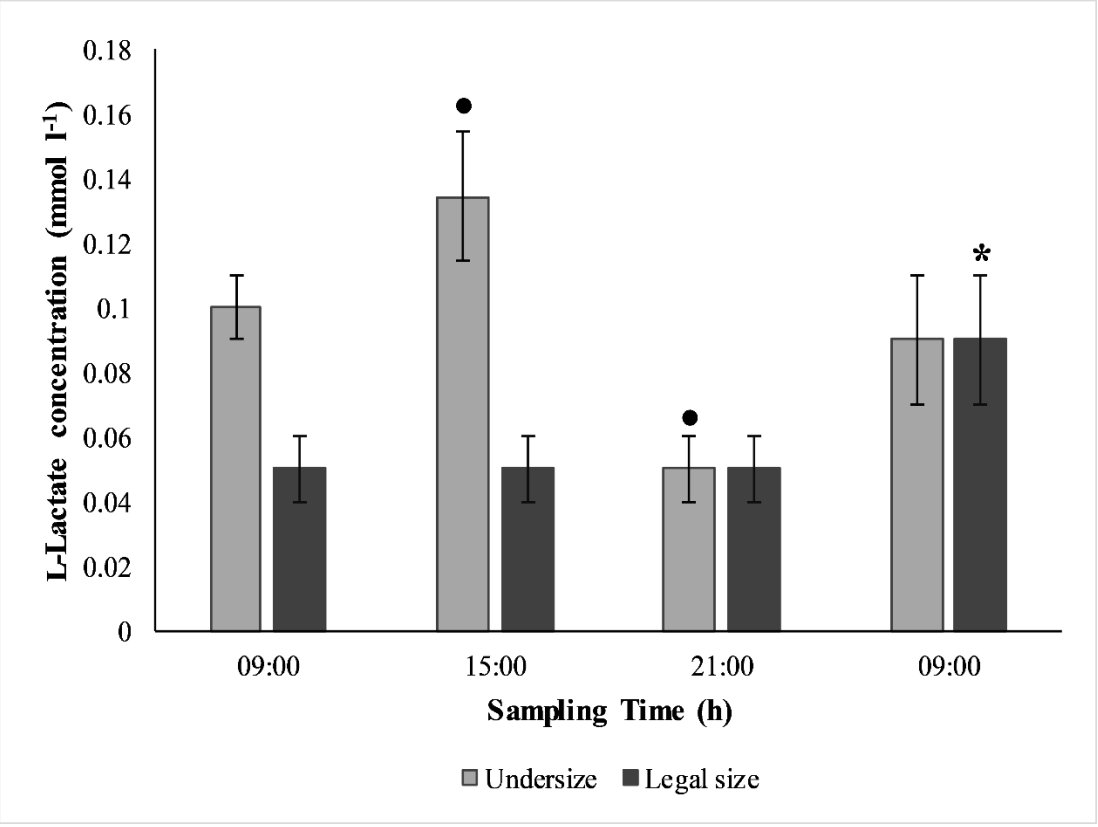


Figure 3.5. Haemolymph L-Lactate concentrations of undersize (< 88 mm) and legal size (≥ 88 mm) *Homarus gammarus* over a 24-hour period. Significance levels between sampling times from Wilcoxon signed ranks test shown as * $p < 0.01$, • $p < 0.05$. Undersize N=13, legal size N=19.

3.4 Discussion

The crepuscular movement of lobsters in this study, along with the peak movement falling within the first few hours after sunset, are in line with previous studies of both *H. gammarus* (Smith *et al*., 1998, 1999) and *H. americanus* (Lawton, 1987; Karnofsky *et al*., 1989; Jury *et al*., 2005) conducted in the field and laboratory. It has been widely accepted in the past that *H. gammarus* are nocturnally active, however the present work, building upon previous studies, has also confirmed that lobsters show a high degree of individual variability (Jury *et al.,* 2005). On the other hand, the closely related American lobster (*Homarus americanus*) alternate nocturnal and diurnal behaviour patterns, with greater periods of activity occurring during the day (Golet *et al.,* 2006). *H. gammarus* are known to possess endogenous rhythmicity as is evidenced in Jury *et al.* (2005) when there were clear daily rhythms of activity with significant increases occurring at night, however, the same study also showed a high degree of variation in individual expression of these patterns under normal conditions. Seasonality has also been shown to influence activity within this species with a positive correlation occurring between decreasing temperatures and a subsequent decrease in movement (Smith *et al.,* 1998). The increase in activity throughout the evening and night has been reported for other crustaceans inhabiting similar benthic areas such as *C. pagurus* (Scott *et al.,* 2018b). Nocturnal foraging which has been documented for both of these species (Seed, 1969; Skajaa *et al.*, 1998; Karnofsky *et al.,* 1989) may be a means of avoiding visually receptive predators including cod, birds, seals, wolffish, and other crustaceans (Rae, 1967, 1968; Rae and Shelton, 1982; Van Der Meeren, 1993; Skajaa *et al.,* 1998). In other crustacean species such as *Carcinus maenas* , nocturnal feeding widens the food spectrum and results in higher pursuit success (Naylor, 1960), and this may also be the case for lobsters.

Activity levels in immature *H. gammarus* has been shown to vary with size, with relative activity of smaller individuals being higher than larger individuals (Mehrtens *et al.,* 2005), but this trend does not appear to exist in adult lobsters. In adult *H. americanus*, Jury *et al*. (2005) did not find a significant difference in distances moved per day by size, which corresponds to the results found in this study.

Natural nocturnal movements and peak in activity after sunset confirmed in this laboratory-based study are an important baseline for any future laboratory or field work that investigates the effects of stressors on lobster behaviour. When comparing past and future results, it is important that time in relation to sunset is considered.

The haemolymph parameters found in this study show variability and diel patterns over the 24-h sampling period, with a few exceptions. These diel patterns (as previously found in *Cancer pagurus* (Scott *et al.*, 2018b) highlight the requirement of baseline measurements for individual species, to avoid generalising across all crustaceans and individuals.

Crustacean haemolymph contains many proteins including Haemocyanin which is the primary oxygen carrying protein in crustaceans (Hagerman *et al.,* 1990). Haemocyanin has been shown to increase during periods of hypoxia (Hagerman *et al.,* 1990) and is also linked with L-Lactate, which causes an increase in O_2 affinity with increased concentrations of L-Lactate (Sanders and Childress, 1992). As expected for a baseline, haemolymph density (1014–1116 g l^{-1}, 1074 ± 2.2 mean ± SE) and haemolymph Haemocyanin levels (15.9–123.7 mg ml^{-1}, 66.7 ± 3.1 mean ± SE) for *H. gammarus* in this study did not vary over the 24 h sampling period. The significantly higher haemolymph density, and close to significant Haemocyanin concentrations, found in males throughout this study is the first recorded case in *H. gammarus* to the authors knowledge. The same sexual dimorphism has been found in the Haemocyanin concentrations of blue crab, *Calinectes sapidus* (Horn and Kerr, 1969), and in spiny lobster, *Palinurus elephas* where males also exhibited higher concentrations (Bellelli *et al.,* 1988).

The results obtained throughout this study correspond well to those in the literature for European and American lobsters. *H. americanus* haemolymph density has been found to vary between 1052.0 ± 7.4 g l^{-1} (Lorenzon *et al*., 2011) and 1053.4 ± 11.4 g l^{-1} (Lorenzon *et al.,* 2007). Zeis *et al.* (1992) measured the concentration of Haemocyanin in *H. gammarus* haemolymph as 37.8 ± 10.3 mg ml^{-1}. Well-fed juvenile *H. gammarus* in Hagerman's (1983) study had mean Haemocyanin content of 22.2–59.2 mg ml^{-1} (0.3–0.8 mM). Higher levels (116.40 mg ml^{-1}) of haemolymph protein concentrations have been found in *Panulirus longipes* (Dall, 1974), this is thought to have been attributed to the

fact that there are seasonal changes in Haemocyanin concentrations in this species with 2-3 times higher concentrations observed in winter (Bellelli *et al.,* 1988).

Haemocytes play important roles in immune responses of crustaceans (Johansson *et al.,* 2000). There are several classifications of haemocyte cells including: hyaline cells (involved in phagocytosis), semi-granular cells (involved in encapsulation), and granular cells (involved in the release of prophenoloxidase) (Johansson *et al.,* 2000). For this study the Haemocyte cells were combined during measurement and had a range of 8.6–121.8 × 10^6 ml^{-1}, (52.7 ± 2.2 × 10^6 ml^{-1} mean SE). These values overlap with results in the literature; however, the average THC from both undersize lobsters (54.19 ± 5.1 × 10^6 ml^{-1}) and legal size lobsters (54.33 ± 4.2 × 10^6 ml^{-1}) are higher, in comparison to those of other crustacean species in the literature (e.g. Jussila *et al.,* 1997, 2001; Hernroth *et al.,* 2004; Celi *et al.,* 2014; Simon *et al.,* 2016; Powell *et al.,* 2017; Ooi *et al.,* 2019). This highlights the need for species-specific measurements of factors previously thought of as relatively constant across several crustacean species.

Values observed in the closely related *H. americanus* ranged from 11 – 28 × 10^6 ml^{-1} in healthy individuals (Cornick and Stewart, 1978) with slightly higher values of 30 × 10^6 ml^{-1} or 37.4 × 10^6 ml^{-1} recorded in infected individuals (Battison *et al*., 2014; Clark *et al*., 2013). These higher values however are in line with baseline values of the Indian spiny lobster (*Panulirus homarus*) (ca. 40 × 10^6 ml^{-1}) (Verghese *et al*., 2007) which further suggest that changes in individuals of a certain species will provide more reliable data than comparison against general crustacean values.

D-Glucose, the primary fuel for ATP production in crustaceans is essential for maintaining metabolic processes (Lorenzon *et al.,* 2011). It has been shown to have a positive correlation with increased locomotor activity (Kallen *et al*., 1988; Kallen *et al*., 1990; Tilden *et al.,* 2001; Lorenzon *et al.,* 2007; Barrento *et al.,* 2010; Barrento *et al.,* 2011) with activity level expected to be reflected in D-Glucose concentrations (Briffa and Elwood, 2001).

In haemolymph *C. pagurus,* D-Glucose concentration increases throughout the day with highest concentrations occurring during peak activity (Scott *et al.,* 2018b). In this study a similar trend was found to exist in *H. gammarus* with D-Glucose concentrations steadily rising in line with increasing activity. The results are not as clear cut as those found for

C. pagurus, primarily due to increased periods of activity during the day in *H. gammarus* which was absent in the crabs.

D-Glucose and L-Lactate has been shown to be linked in a circadian rhythm in other crustacean species, resulting in a rise in D-Glucose throughout the day and a subsequent decrease in L-Lactate (Scott *et al.*, 2018a). A direct correlation was not immediately apparent in *H. gammarus* although an increase was seen throughout the day in D-Glucose. This potentially suggests a circadian rhythm lasting longer or shorter than 24 h, or a lack of a circadian rhythm altogether. Upon closer inspection a slight decrease did occur throughout the day in L-Lactate in most individuals, although this was not deemed significantly different. D-Glucose concentrations show high individual variability in *H. gammarus*.

D-Glucose values obtained throughout this experiment (0.0307–1.542, 0.458 ± 0.0244 mmol l^{-1}) correspond with those found in the literature for other lobster species. *H. americanus* was found to have a mean range of 0.68 ± 0.19 to 2.04 ± 0.83 mmol l^{-1} D-Glucose concentration, dependent on the body temperature of the individual (Lorenzon *et al.*, 2007). A study by Radford *et al.* (2005) found that baseline haemolymph glucose concentrations of juvenile rock lobsters (*Jasus edwardsii*) was 0.61 ± 0.02 mmol l^{-1}; whereas, Spanoghe and Bourne (1999) reported mean D-Glucose concentrations ranging from 0.16 ± 0.05 to 1.25 ± 1.12 mmol l^{-1} in spiny lobster (*Palinurus cygnus*). D-Glucose values for other crustaceans fall into a similar range including: 0.09 to 1.40 mmol l^{-1} in edible crab, *C. pagurus* (Scott *et al*., 2018b), 0.77 to 1.39 mmol l^{-1} in the giant tiger prawn (*Panaeus monodon*) (Hall and Van Ham, 1998), 0.9 ± 0.2 mmol l^{-1} in the freshwater crayfish *Procambarus clarkii* (Garcia *et al*., 1993), 0.49 mmol l^{-1} in Jonah crab (*Cancer borealis*) (Glowik *et al.*, 1997), and 0.209 ± 0.018 mmol l^{-1} in the spider crab (*Maia squinado*) (Durand *et al.*, 2000).

L-Lactate, as a metabolite in crustaceans is typically an indicator of anaerobic respiration due to hypoxic/anoxic conditions or impaired gill function (Durand *et al.*, 2000). As with D-Glucose concentrations there was a degree of high individual variability which makes diel changes more difficult to detect at a baseline level for this species. L-Lactate showed decreases throughout the day, which although not significant still highlights that a circadian rhythm exists with a link between this parameter and D-Glucose. The high individual variability in L-Lactate creates outliers within the data which may blur the

significance that would be expected during daytime sampling. L-Lactate can also be underrepresented in haemolymph samples, as it may compartmentalised in other tissues (Full and Herreid II, 1984; Ocampo *et al*., 2003).

L-Lactate concentrations found in this study (0.00466–0.347 mmol l^{-1}, 0.0749 ± 0.00578 mmol l^{-1}) correspond with those found in the literature for *H. gammarus* at 0.9 ± 0.1 mmol l^{-1} (Taylor and Whiteley, 1989) and 0.5 ± 0.2 mmol l^{-1} (Zeis *et al.*, 1992), *C. pagurus* at 0.03 to 12.65 mmol l^{-1} (Scott *et al*., 2018b) and 1.22 ± 0.47 to 2.23 ± 0.59 mmol l^{-1} (Scott *et al*., 2018a), and *M. squinado* at 0.164 ± 0.013 mmol l^{-1} (Durand *et al.*, 2000).

The use of L-Lactate and D-Glucose as a stress component in future research must factor in individual variability and the presence of circadian rhythms to accurately determine potential affecting factors. Haemocyanin concentrations and haemolymph densities which show differences between male and females must also be factored into future research on this species. Seasonality may have implications on physiological stress parameters within this species and warrants future investigation. The activity level of this species may also vary significantly depending on the time of year, much as it does on the time of day. The results obtained from this study represent *H. gammarus* from a particular location at a particular time of year, further work is required to determine geographical and seasonal changes. Baseline physiological and behavioural data is essential when investigating the effects of anthropogenic stressors on organisms, however, is currently lacking in the literature. This chapter highlights the diel changes in haemolymph parameters, the crepuscular changes in activity level and the sex differences in haemolymph density in *H. gammarus*. These types of variations across many different species could prove problematic when assessing the effects of stressors and highlight the importance of baseline data.

Chapter 4. Understanding the effects of electromagnetic field emissions from Marine Renewable Energy Devices (MREDs) on the commercially important edible crab, *Cancer pagurus* (L.)

4.1 Introduction

The predicted decline in non-renewable energy sources in future decades (Pimentel *et al.*, 2002) indicates the need for alternative renewable energy sources. Due to reduced planning constraints, lack of inexpensive land near major population centres (Bilgili *et al.*, 2011), and perceived aesthetic problems with many renewable energy structures (Gill, 2005), there is increasing pressure to move potential locations offshore. Wind speeds tend to be significantly higher offshore than onshore thus producing larger amounts of energy per turbine (Bilgili *et al.*, 2011). Vast open spaces offshore also help avoid wake effects (shading effect of a turbine on those downwind of it) by allowing turbines to be placed at greater distances apart (Chowdhury *et al.*, 2012). As the global energy demand grows, inshore areas are increasingly being utilised by the energy sector looking to increase energy production via wave and tidal energy devices (Frid *et al.*, 2012). Therefore, there is a requirement for appropriate assessment of the implications of both offshore and inshore renewable energy generation with regards to current ecological status and potential future consequences (Gill, 2005). Currently, the UK is the largest global producer of electricity from offshore wind farms and has more projects in planning or construction than any other country (Smith *et al.*, 1999; Crown Estates, 2017). Proposed sites and developments are based on current knowledge and assessments of the local environment, despite relatively little being known about the ecological effects of such developments on marine benthic organisms. Some studies suggest that turbine arrays could increase biodiversity through new habitat provision (Landers *et al.*, 2001; Lindeboom *et al.*, 2011), whereas detrimental effects of turbine arrays on birds (Garthe and Hüppop, 2004) and fish (Westerberg and Lagenfelt 2008) have also been found. Furthermore, it is feared that marine mammals might be sensitive to minor changes in magnetic fields associated with these developments (Walker *et al.*, 2003). There is currently a gap in our knowledge of the effects of these arrays on crustaceans.

Electromagnetic fields (EMF) are associated with Marine Renewable Energy Devices (MREDs). EMFs originate from both anthropogenic (telecommunication cables, power cables, marine renewable energy devices) and natural (Earth's natural geomagnetic field) sources. It has been shown that industry-standard AC cables can be effectively insulated to prevent electric field (E- field) emissions but not magnetic field (B-field) emissions (Gill, 2005). Standard cable configurations combined with the existing B-field emission creates induced electromagnetic fields (iEMF) (Gill, 2005). The magnetic field (B- field) leakage has been shown to be of concern as it will interact with magnetite-based internal compasses in marine organisms (Öhman *et al.,* 2007). Electric currents between 850 and 1600 Amperes (A) tend to be found in undersea cables consequently producing an electromagnetic field of around 3.20 millitesla (mT) (1,600A) in a perfect wire (Bochert and Zettler, 2006). As with all electromagnetic fields this quickly diminishes away from the source, with values of around 0.32mT and 0.11mT at 1 metre (m) and 4 m respectively (Bochert and Zettler 2006). In a report by Normandeau *et al.* (2011) there was shown to be a great variation in electromagnetic field strength arising from different structures, cables and current values. In a recent report (Thomsen *et al.,* 2015) higher EMF emission values were recorded for export cables compared to inter turbine cables. It was also noted in this report that EMF values recorded were considerably higher around the cables than around the wind turbine bases. An assessment of the literature (COWRIE, 2003) highlights that the current state of knowledge on EMF strengths emitted by undersea power cables is insufficient to allow an informed assessment.

The European edible crab, *Cancer pagurus* is found throughout Western Europe from Norway to northern France. They are commonly found from the shoreline to offshore depths around 90m. They are a heavily exploited commercial species with the present UK and Ireland annual catch around 34,600 tonnes (Bannister 2009). There is a high probability that this species will encounter sub- sea power cables resulting in increased EMF exposures, potentially leading to stress responses. In crustaceans, haemolymph analysis enables measurement of stress through detection of abnormalities in internal chemical processes. Previous studies (Taylor *et al.,* 1997; Durand *et al.,* 2000; Bergmann *et al.,* 2001; Lorenzon *et al.,* 2007) show that L-Lactate and D-Glucose are useful measures of stress in crustaceans, whilst respiration rates in marine organisms

are also reliable indicators of certain environmental stressors (Paterson and Spanoghe 1997; Doney *et al.,* 2012; Brown *et al.,* 2013). It is also known that behavioural and response parameters (attraction/avoidance, antennular flicking rate, and activity level) can be affected by stress (Stoner, 2012). The aim of the present chapter is to determine the effects of EMFs on edible crabs using a combination of the above stress indicators.

4.2 Materials and Methods

Intermoult crabs were obtained from local fishermen and the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK) for each experiment. Crabs were kept in 1000 L flow through tanks with ambient sea temperature and natural photoperiod for a minimum acclimation period of 1 week at densities of no more than 5 crabs per tank. Each crab was sexed, carapace width measured (mm) and a condition assigned using a condition index (Table 4.1). Crabs were categorized into size classes based on carapace width (10-79 mm – small, 80-120 mm – medium, 121 mm+ - large). Experimentation was conducted in July – August 2017.

Table 4.1. Condition index for *Cancer pagurus*. All crabs used throughout these experiments were grade 1 or 2 (Adapted from Haig *et al.,* 2015)

Index	Description
1 – Perfect	Body intact with no damage, black spot or other visible defects.
2 – Good	One or two legs missing no carapace damage.
3 – Ok	More than two legs missing, limited carapace damage or slight blackspot.
4 – Poor	One or both claws missing, damaged carapace and widespread blackspot.
5 - Bad	Legs and claws missing, extensive carapace damage and/or blackspot.

Physiological analysis

Haemolymph analysis

During experimentation four 70 L tanks were set up with flow through seawater (UV sterilised and filtered) which was temperature controlled (TECO TK1000) to ambient conditions. Temperature and light intensity were constantly measured via data loggers

(Onset HOBO temperature/light pendant). Within each tank a perforated plastic enclosure enabled the crab to be held in position over the magnets. The EMF was produced by placing four electric solenoid magnets (24V) connected to variable power supplies (QW-MS305D) on ceramic tiles underneath the tanks. The magnets were run at full power, thus creating an electromagnetic field (peak 40mT measured by an AlphaLab, Inc Gaussmeter Model GM-2) which covered the experimental area. The experiment was repeated using a lower strength EMF (peak 2.8mT) to correspond with the expected, although highly variable, levels on the surface of a sub-sea power cable and correspond to those in previous studies (Bochert and Zettler, 2006). Haemolymph samples were collected, within 60 seconds, from the arthrodial membrane at the base of the fifth walking leg using 1 ml syringes with 25G needles. Samples of 250 µl, 300 µl and 700 µl were collected from the different size groups respectively. Haemolymph was transferred into 1.5 ml cryogenic vials, with 50 µl of haemolymph from each sample stored in a separate vial for Haemocyanin analysis. Samples were frozen in liquid Nitrogen and stored in a freezer (-25°C). To obtain baseline data, haemolymph was collected before exposure (0 h) then again after 4 h, 8 h and 24 h. All haemolymph collection was staggered with 5 mins between each sample to ensure time consistency throughout the experiment. For experiments, sample times were as follows: 0 h (09:00), 2 h (11:00), 4 h (13:00), 6 h (15:00), 8 h (17:00) and 24 h (09:00).

Haemolymph was deproteinated using the procedure of Paterson and Spanoghe (1997). Samples were thawed, vortexed and mixed with an equal volume of chilled 0.6 M perchloric acid (BDH 10175). Inactivated proteins were separated by centrifugation at 25,000g for 10 mins (Eppendorf 5417C, rotor 30 x 1.5-2ml). After neutralizing the supernatant with 3 M potassium hydroxide (BDH 29628) the precipitated potassium perchlorate was separated by centrifuging at 25,000g for a further 10 min. The supernatant was then frozen and stored at -25°C.

D-Glucose

D-Glucose concentration was measured using a D-Glucose assay kit (Sigma GAGO20-1KT) according to the procedure in Barrento *et al.* (2010). Haemolymph samples were incubated for 30 min at 37°C with double the assay reagent. 300 µl of 12N sulphuric acid (BDH) was added to stop the reaction and the solution added to a 96 well flat-bottomed microplate (Wheaton 712122). The plates were then analysed

spectrophotometrically at 540nm (Molecular Devices, Spectramax M5) and D-Glucose concentration calculated using a calibration curve of standards with a known concentration.

L-Lactate

L-Lactate concentration of deproteinated haemolymph samples were measured using L-Lactate reagent (Trinity Biotech, Wicklow, Ireland no. 735-10), per the procedure described by Barrento *et al.* (2010). Samples of haemolymph (2.8 µl) were mixed with L-Lactate reagent (280 µl), then incubated for 10 min at room temperature. These were then added into the wells of a 96-well flat-bottom microplate. The plate was then analysed spectrophotometrically at 540nm and L-Lactate concentration was calculated from a calibration curve using standards of known concentration (Trinity Biotech, Wicklow, Ireland L-Lactate standards set no. 735-11).

Haemocyanin

Haemocyanin concentrations were determined spectrophotometrically. 50 µl of haemolymph was diluted with 2ml chilled distilled water, 280 µL was added to the wells of the 96-well flat-bottom microplate and the absorbance at 335nm was measured twice. Haemocyanin concentration (mg/ml) was calculated from the molar extinction coefficient $E_{1\ cm}^{mM} = 17.26$, as previously described by Harris and Andrews (2005).

Respiration

Thirty juvenile (≤79 mm carapace width) intermoult crabs were collected from the intertidal zone around St Abbs and placed into two 1000 L tanks with seawater flow-through. Crabs with a carapace width of over 80 mm were too large for the respiration chamber so were discarded. Inside a Helmholtz coil (2.8mT) a 46 L flow through tank was set up as a water bath, with filtered, UV sterilised seawater connected to a sump tank and temperature control unit to ensure temperature stability. A 0.3 L respiration chamber was filled with UV sterilised filtered seawater and placed into the water bath. The fibre optic probe (Presens polymer optical fibre POF) was attached to the chamber. An optical oxygen meter (Presens Fibox 3) was used to measure oxygen levels using Presens PSt3 (detection limit 15ppb) sensor spots. This meter was connected to a computer and a blank was run for a period of 30 min. To eliminate bacterial respiration from water samples, a blank was run prior to each trial and the information obtained

was considered when calculating oxygen consumption of the crabs. The system was calibrated using a conventional two-point oxygen-free and oxygen-saturated system. Oxygen-free water was obtained using Sodium sulphite (Na2SO3) to remove any oxygen, whilst oxygen saturation was achieved through bubbling air vigorously into the water sample for a period of 20 min, stirring to ensure the water was not supersaturated.

Crabs were randomly selected, weighed and carapace width measured. The crabs were then placed into the respiration chamber and acclimated for 1 h with the water flow-through valve open. After acclimation, the valve was closed and measurements taken until a limit of 60% air saturation was reached, or for a total of 30 min. Fifteen individuals were ran as control with the Helmholtz coil switched off and 15 were acclimated with no EMF present then subjected to an EMF for the duration of the experiment. The % air saturation was recorded for each individual and converted to oxygen consumption (mg/g/h).

Helmholtz Coil

Two Helmholtz coils were set up with four 12 L glass tanks each, situated in a recirculated temperature-controlled water bath. Tank sides were covered with netting to reduce visual stimuli. Tanks were kept at 10°C and were constantly aerated with air stones. Ten large male and 10 large female crabs were randomly selected (carapace width 121 mm+), weighed and carapace width recorded before being placed into the experimental tanks. After a 1 h acclimation period, baseline haemolymph samples were taken from each crab (800 µL) and one of the Helmholtz coils switched on, with the other acting as a control. Subsequent haemolymph samples were taken at 2, 4 and 6 h. Haemolymph was sampled using the previously mentioned protocol. After 6 hours, the Helmholtz coil was switched off and the crabs were left overnight. 24 h after the baseline haemolymph sample was taken another baseline sample was taken and the other Helmholtz coil was switched on and further samples taken at the same times as the previous day. This allowed all crabs to be sampled during exposure to EMF and control conditions and helped to eliminate individual variances by comparing an individual throughout both treatments. The EMF created by the Helmholtz coil was measured and mapped and gave a field strength of 2.8mT uniformly distributed throughout the experimental area. Three additional individual crabs were sampled over the two-day experiment with no exposure to EMF to account for any handling stress.

No elevated stress levels were detected. The aims of these trials were to detect any changes in haemolymph parameters over a shorter period of time. Large crabs were utilised to allow larger volumes of haemolymph to be sampled over a short time frame.

Behavioural analysis

Antennular flicking rate

A 12 L glass tank was set up with a 40 L sump tank containing UV sterilised filtered sea water that was temperature controlled (TECO TK1000) to 12°C. The experimental tank was placed on top of 4 solenoid electromagnets to create an EMF of 2.8mT. The inflow and outflow were separated from the crab inside the tank by inserting a perforated plastic sheet to reduce visual disturbance. Experimental tanks were placed behind screens to avoid external stimuli. Crabs were acclimated to the experimental tanks for 30 minutes prior to testing after which the camera was set to record via a remote control. The crab was recorded for 10 min under control conditions, then a further 10 min with an EMF present. After each trial, the tanks were sterilised and underwent a full water change to reduce chemical cues which may affect antennular flicking rates. The system was monitored for temperature, dissolved oxygen and salinity during all trials.

The video data was post-processed with flicking rate counted for both antennules by a minimum of 3 trained people per video file for accuracy. Trials where the crab was asleep or did not exhibit any antennular flicking were discarded.

Activity and side selection

Four 70 L experimental tanks were set up and connected to a 1000 L temperature-controlled sump tank which received a constant supply of UV sterilised filtered sea water. The sides of the experimental tanks were shaded to reduce visual disturbances. A wide aperture mesh was placed over the top of the tanks to prevent the crabs from escaping. Water was pumped from the sump tank into the four experimental tanks at an equal rate for the duration of the experiment and the temperature was constantly monitored using data loggers (Onset HOBO). After each trial the tanks were drained, sterilised (Virkon aquatic) and refilled.

Four waterproof Infrared cameras were suspended above the experimental tanks and set to record during each trial. The trials consisted of:

1. Day conditions – (7 hours 30 minutes (08:30am-16:00pm)

2. Night conditions – (8 hours (20:00pm- 04:00am)

The footage from each tank was post processed then analysed using Solomon Coder (version – beta 17.03.22). Each video file was broken down to still images at 1 min intervals for the duration of the trial. The position of the crab in each image was analysed and a movement index was created by assigning a value of 0 to a picture where there was no movement, when compared to the previous picture, and a value of 1 where there was movement. The total movement index score was recorded for each tank throughout all the trials and used to indicate activity levels in the crabs. The individual pictures were analysed to determine the percentage of time each crab spent on either side of the tank (magnet or non-magnet). This was used to indicate an attraction to or avoidance of the EMF. Trials where there was no movement for the entire duration or the crab did not experience both sides of the tank were omitted. This was deemed necessary as the individual would not be making a choice based on treatment preference. It was concluded during preliminary trials that the crabs spent a significant amount of time in the corners of the tanks (approx. 85%), thus influencing magnet placement.

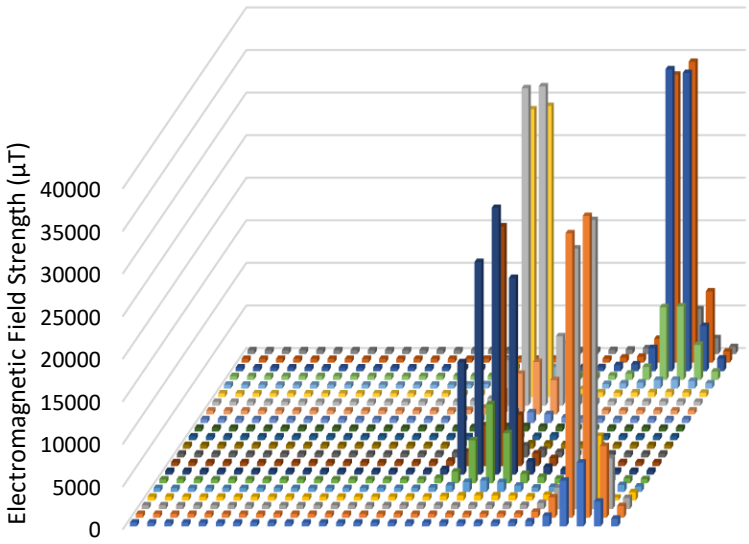


Figure 4.1. Electromagnetic field strength (µT) over the tank floor (square inch) represented by the x axis. Quad magnet set-up with the corner magnets plus an additional two solenoid magnets placed just offset to create an EMF over half of the tank. Magnets were swapped randomly from the left to right sides of the tank during replication.

In the set-up the magnets were evenly spaced in the middle of one side of the tank in addition to the two magnets in the corners. Experiments were conducted under day and night conditions to fully assess the behaviour of this crepuscular species. In control conditions the magnets were present but not switched on. Magnet placement (left or right) was randomised to reduce any tank based or external stimuli that may affect results. The EMF was mapped for the setup using a 1sq. inch grid over the base of the tank with each square being measured by an AlphaLab, Inc Gaussmeter Model GM-2 (Figure 4.1.).

Shelter selection

To further determine the effects of EMFs on crab behaviour and potential attraction, four 70 L experimental tanks were set up with temperature controlled (13°C), flow through UV sterilised seawater (Figure 4.2.). Six black ABS plastic shelters (300 mm x 200 mm x 100 mm) were constructed and secured to the bottom of the tanks. In two of the tanks two plastic shelters were set up, with four solenoid electromagnets placed under each shelter. During each trial one of the shelters' electromagnets would be turned on with the other remaining off as a control. In the two remaining tanks a single shelter was set up with four solenoid electromagnets under each, one tank having the magnets switched on and the other remaining off as a control. All magnets were set so that an EMF of 2.8mT was present under the length of the shelter. An individual large crab (121+ mm carapace width) was placed into each tank, using an even split of male and females. Using the same infrared camera set-up previously described, the crabs were recorded from 23:00pm – 06:00am and the video files post-analysed (Solomon Coder) to determine the percentage of time spent in the shelters or free roaming within the tank. The primary purpose of setting up single shelter tanks was to determine how the crab would interact with the shelter under control conditions, and to determine how the crab would act if the only shelter available is subjected to EMF. The dual shelter tanks were set up to determine if there was an attraction to EMFs and to discover how crabs would split their time between seeking shelter and active roaming.

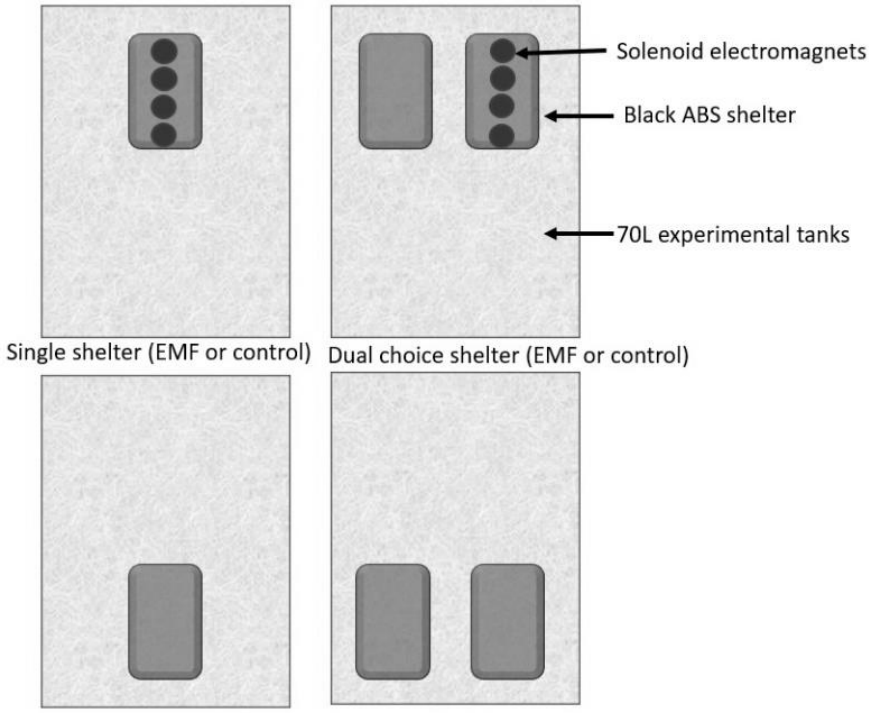


Figure 4.2. The four different shelter experimental tanks. Each tank had 4 solenoid electromagnets underneath each shelter. The shelter with the electromagnets turned on was randomised along with the position of each tank to remove experimental bias and potential external variable factors. (EMF = magnets on, Control = magnets off).

Statistical analysis

Results were expressed as mean ± standard error (SEM). When data met ANOVA assumptions (per Shapiro-Wilk test for normality and Levene's test for equality of error variances) multiple-comparison tests (paired t-test, one-way ANOVA, 2-way ANOVA) were conducted to reveal differences between groups. If data could not meet ANOVA assumptions, non-parametrical analysis (Wilcoxon signed rank test, Mann-Whitney, Scheirer-Ray-Hare) was performed. Chi-square test (2 tailed) was utilised for choice experiments. Post-hoc analysis for parametric data (Tukey's test) and non-parametric (pairwise Mann-Whitney) were conducted. All statistics were tested at a probability of 0.05 (IBM SPSS Statistics v.23 SPSS Corp. Chicago, USA).

4.3 Results

Physiological analysis

Haemolymph parameters

Exposure to EMF had a significant effect on the L-Lactate levels of *Cancer pagurus* (Table 4.2.). Throughout the 24-h high strength (40mT) exposure L-Lactate levels followed the same circadian rhythm as the control group, with a gradual decrease in concentration throughout the day before a rise at night (Figure 4.3.). Despite following the same patterns, the EMF exposed values were significantly lower at 4 h ($Z=2.69$, $p<0.05$, Wilcoxon signed rank test) and 8 h ($Z=2.74$, $p<0.05$, Wilcoxon signed rank test) when compared to 0 h. The control group showed a decrease in concentration throughout the day, however, there were no significant differences between the baseline sample and the remaining samples taken over the 24 h. Exposure to low strength EMF (2.8mT) disrupted the natural circadian rhythm of L-Lactate causing a significant decrease throughout the 24-h trial. The typical rise and peak values normally obtained at dawn were absent. The different EMF strengths had a significant effect on L-Lactate level ($Z=2.46$, $p<0.05$, Mann Whitney U-test). After 4 h of exposure crabs exposed to the high strength EMF had significantly lower concentrations of L-Lactate compared to those in low strength EMF. D-Glucose levels showed a significant increase between 0 h and 4 h, 0 h and 8 h in control crabs ($Z=2.59$, $p<0.05$, $Z=2.13$, $p<0.05$, Wilcoxon signed rank test) and in crabs exposed to 40mT EMF ($Z=2.90$, $p<0.01$, $Z=2.67$, $p<0.05$, Wilcoxon signed rank test) (Figure 4.4.). Crabs exposed to 2.8mT EMF did not show the significant rise in D-Glucose levels after 8h of exposure. Haemolymph D-Glucose levels of low and high strength EMF exposed crabs followed very similar daily patterns. Although D-Glucose concentrations after 4 h and 8 h were lower in exposed crabs compared to control, the difference was not statistically significant. Exposure had no effect on the remaining haemolymph parameters. Haemocyanin levels remained constant (44.08 ± 1.01 mg/ml) throughout the trials, with no significant variation over time or by crab size.

Table 4.2. L-Lactate and D-Glucose concentrations (mM) for the Helmholtz (2.8mT) trials (Mean ± SEM).

	Helmholtz			
	L-Lactate		D-Glucose	
	EMF	Control	EMF	Control
0h	1.21±0.33	2.23±0.59	0.24±0.04	0.31±0.06
2h	1.35±0.25	1.81±0.45	0.47±0.04	0.46±0.06
4h	1.05±0.22	1.47±0.39	0.72±0.7	0.73±0.07
6h	1.03±0.23	1.22±0.47	0.81±0.08	0.71±0.08
N	20	20	20	20

To test whether EMF had any effect on the measured haemolymph stress parameters after exposure, half of the crabs used in the Helmholtz trials were sampled the following day at 0, 2, 4 and 6 h. Exposure to EMF has no lasting effects on the haemolymph stress parameters. The increase in EMF strength from 2.8mT to 40mT had no effect on the D-Glucose or Haemocyanin parameters, but showed an overall decrease in mean L-Lactate concentration throughout the sample group. This change in concentration could potentially be explained by high individual variability or limits of the assay kit used.

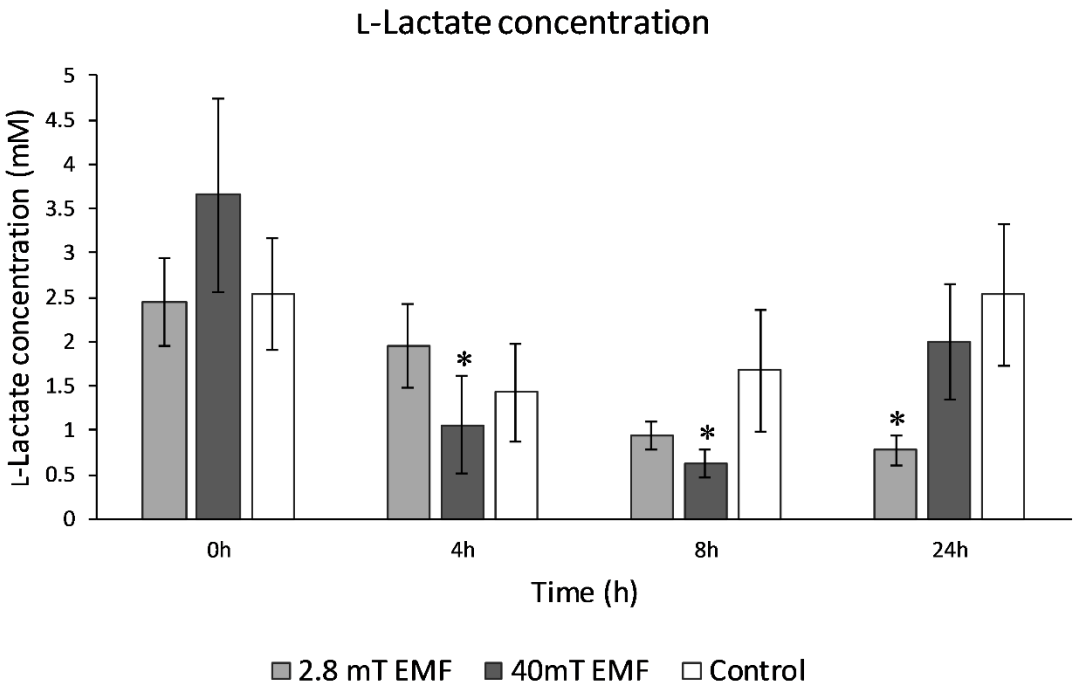


Figure 4.3. L-Lactate over a 24-h period during control conditions and exposure to low strength (2.8mT) and high strength (40mT) EMF starting at 9:00am. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). The L-Lactate circadian rhythm was disrupted by exposure to 2.8mT EMF and did not follow the usual trend, showing a continuous decrease and significantly lower values after 24 h. The L-Lactate circadian rhythm was altered during exposure to 40mT EMF resulting in much lower concentrations after 4 h and 8 h despite following the same trend found in the control results. Values are presented as Mean ± SEM, * is the significance from 0 h within respective treatments ($p<0.05$).

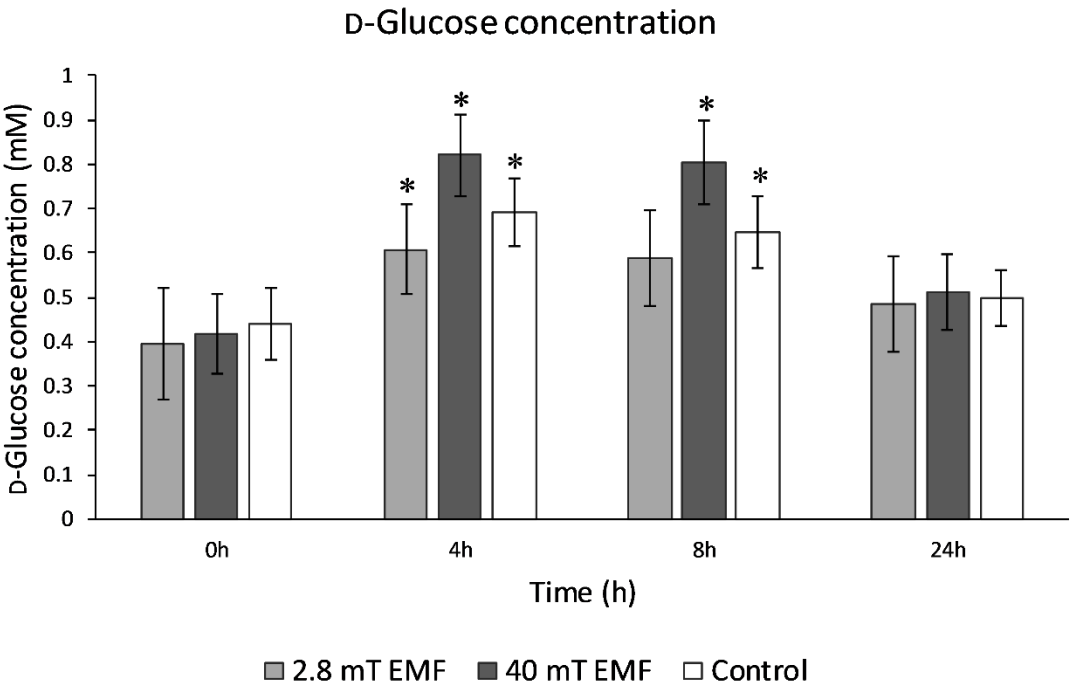


Figure 4.4. D-Glucose concentration over a 24-hour period during control conditions and exposure to low strength (2.8mT) and high strength (40mT) EMF. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). D-Glucose levels followed a similar circadian rhythm in control and 40mT EMF exposed crabs, with a significant increase towards peak locomotor activity, while 2.8mT exposed crabs were lacking this increase and showed no significant elevation after 8 h. Values are presented as Mean ± SEM, * is the significance from 0h within respective treatments ($p<0.05$).

Respiration

The mean respiration rate of juvenile crabs exposed to EMF was 0.05±0.006 mg O_2/g/h, this showed no difference to values obtained from individuals under control conditions. EMF exposure did not increase O_2 demand and appears to cause no oxidative stress.

Behavioural analysis

Flicking rate

Exposure to EMF caused a slight increase in antennular flicking rate in small and medium crabs although this was not statistically significant (Figure 4.5.). The average pre-EMF flicking rate of 22±4 flicks/min remained unchanged during exposure to EMF

(24±4 flicks/min). The mean flicking rate in the first minute of exposure to EMF (25±4 flicks/min) remained unchanged from initial measurements in control conditions (25±4 flicks/min).

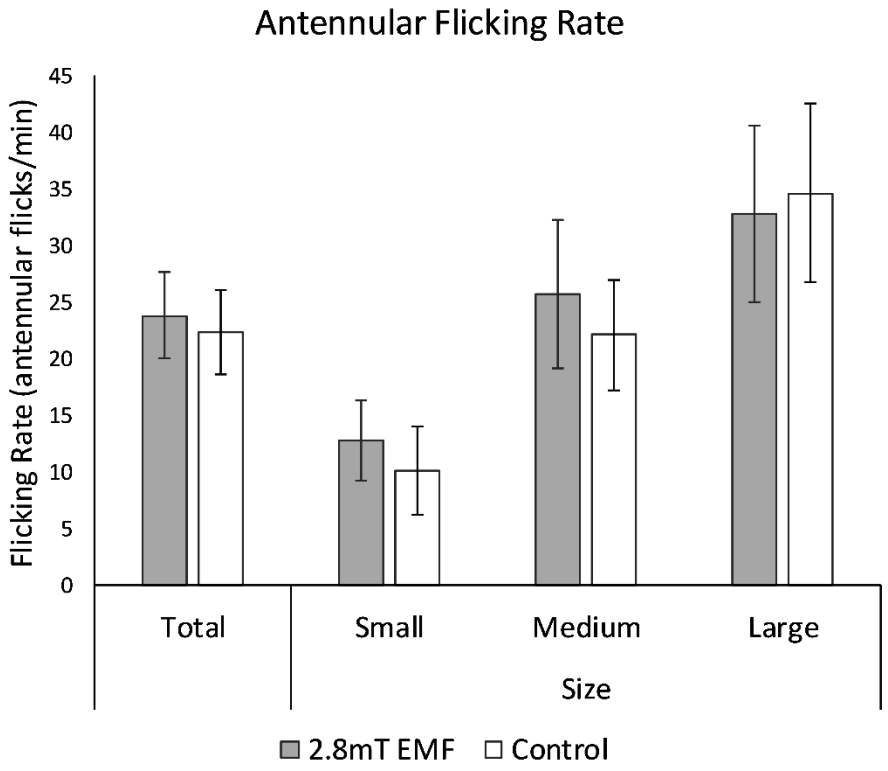


Figure 4.5. Antennular flicking rate (Mean ± SEM), calculated as the mean number of antennular flicks per minute, of individuals exposed to EMF and control conditions for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) (N=30) and combined.

Activity level

During day conditions, there was no significant difference in activity levels between EMF exposed crabs and control, with size being the only significant factor ($F(1,77)=13.43$, $p<0.05$, 2-way ANOVA) (Figure 4.6). The decrease in activity level corresponds with an increase in crab size, with small crabs (19.6±2.5%) having higher activity levels than large crabs (10.1±3.2%). During night conditions, there was a significant increase in activity levels for all size groups ($F(2,114)=4.84$, $p<0.05$, 2-way ANOVA). Small (42.7±5.6%) and medium crabs (45.5±5.1%) had significantly higher activity levels than large crabs (25.9±2%).

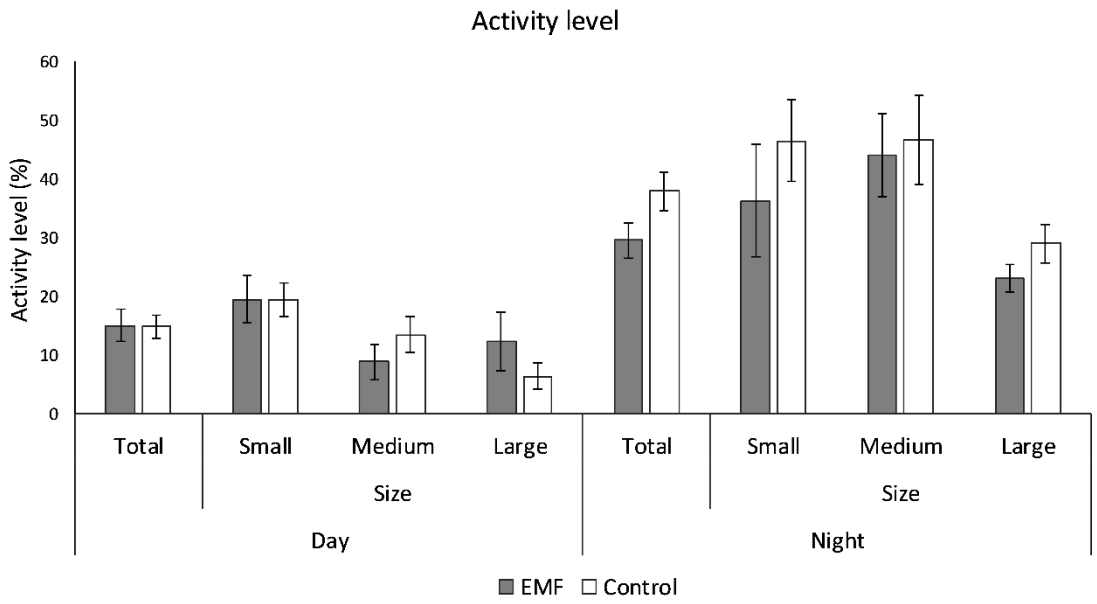


Figure 4.6. Activity level (Mean ± SEM), calculated as percentage movement broken down in to 1 min intervals of the total trial duration, for the three size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm+) in both day and night conditions, along with combined totals. N_{DAY}=79, N_{NIGHT}=117.

Side selection

Under control conditions crabs spent significantly more time on one side of the tank in both day (32-68±5.9%) ($p<0.05$, Chi square test) and night (36-64±3.2%) ($p<0.05$, Chi square test) conditions (N=96). When there was an EMF present there were no clear preferences made between sides during both day (44-56±5.9%) and night (50-50±4.4%) conditions (N=99). There was a significant difference between control and EMF exposed crabs' mean side selection for both day ($p<0.05$, Chi square test) and night ($p<0.05$, Chi square test). This shows that in the presence of an EMF individual crabs fail to make a clear side preference.

Single shelter preference

The mean time spent in the shelter (48%) and out (52%) was approximately equal in the control trials (Figure 4.7.B). When there was an EMF present in the shelter the proportion of mean time spent within the shelter increased to 69% (Figure 4.7.A). These trials also confirmed what was discovered in the dual shelter set ups in that the percentage time spent roaming the tank significantly decreases from 52% in the control

to 31% when there was an EMF present ($p<0.05$, Chi square test). The overall mean percentage time spent in both locations was significantly different between control and EMF conditions ($p<0.001$, Chi square test).

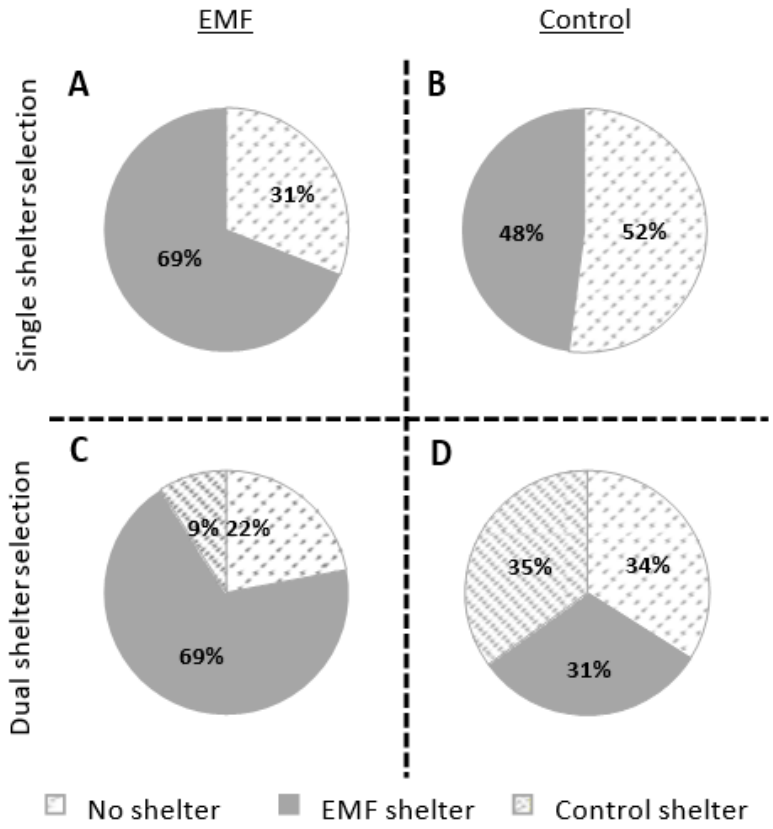


Figure 4.7. Effect of EMF on shelter selection. Time spent in single shelter and outside of the shelter, if exposed to EMF (A) and when the magnets are switched off (B). EMF (N=11) and control (N=11). Time spent in each shelter and outside the shelters, when one of the shelters is exposed to EMF (C) and if none are exposed (D). EMF (N=15) and control (N=15) Shown as a percentage of total trial time (%).

Dual shelter preference

Under control conditions the mean time spent in each shelter and out roaming in the tank were equal (35% EMF shelter, 31% CT shelter and 34% No shelter) (Figure 4.7.D). During EMF exposure, there were clear preferences for the shelter with the EMF present resulting in 69% mean time spent within, with only 9% spent in the control shelter and 22% spent roaming the tank (Figure 4.7.C). There was a drop in mean time from 34% under control conditions to 22% during EMF exposure suggests that once the crabs

detect EMFs they will begin to seek shelter and are drawn to the shelter with the EMF emanating from within. The overall mean percentage time spent in all three locations was significantly different between control and EMF conditions ($t(33)=7.51$, $p<0.001$, t-test). Throughout all shelter trials an equal number of male and female crabs were used. There were no significant differences in behaviour between the sexes.

4.4 Discussion

L-Lactate and D-Glucose both followed a natural circadian rhythm with a rise in D-Glucose, and a subsequent fall in L-Lactate concentrations throughout the day. L-Lactate levels rise throughout the night due to increased activity and subsequent increase in glucose metabolism. In crustaceans, haemolymph glucose and lactate levels are affected by various environmental conditions and stressors (Kallen *et al.,* 1990; Reddy *et al.,* 1996; Chang *et al.,* 1998) and should cycle together under normal, unstressed conditions. L-Lactate is an indicator of anaerobic respiration, typically due to impaired gill function or hypoxic conditions (Durand *et al.,* 2000). D-Glucose, the primary fuel for ATP production in crustaceans is crucial in maintaining metabolic processes (Barrento *et al.,* 2010). D-Glucose levels show a negative correlation with vigour where healthy individuals have lower levels and moribund crabs become hyperglycaemic (Barrento *et al.,* 2010). Activity levels in crabs should partially be reflected in D-Glucose concentrations (Briffa and Elwood, 2001). D-Glucose levels were found to correlate well with current literature in that there was a continual rise in concentration in relation to locomotor activity (Hamann, 1974; Reddy *et al.,* 1981; Kallen *et al.,* 1988; Kallen *et al.,* 1990; Tilden *et al.,* 2001). This suggests that D-Glucose levels continually rise throughout the night until peak locomotor activity has been reached during which the levels will begin to decrease back to original values. EMF exposure did not significantly influence activity level which is consistent with minor changes in D-Glucose levels. Several studies have shown that EMF can alter the circadian rhythm of animals through altering melatonin levels (Reiter, 1993; Schneider *et al.,* 1994; Levine *et al.,* 1995; Wood *et al.,* 1998). Melatonin, a neuropeptide, present in crustaceans, can cause shifts in L-Lactate and D-Glucose cycles (Tilden *et al.,* 2001). This suggests that exposure to a field of 2.8mT could affect melatonin secretion, which consequently alters

L-Lactate and D-Glucose circadian rhythms. At 2.8mT, the L-Lactate concentration follows the circadian rhythm and decreases throughout the day when activity levels are generally lower. During the night there were no differences in activity levels between 2.8mT and control crabs, however there were no increases in L-Lactate levels.

The suppression of the rise in L-Lactate prevents the increase in O_2 affinity of Haemocyanin that would naturally occur (Sanders and Childress, 1992). An increase in L-Lactate has been shown to occur in *Carcinus maenas* during emersion when the crabs would be relying on anaerobic respiration. During re-immersion L-Lactate levels remained elevated after 1 h suggesting that the crabs have to repay an oxygen debt (Simonik and Henry, 2014). Exposure to EMF supresses the rise in L-Lactate which enables the crabs to repay the oxygen debt accrued during periods of higher activity. During long exposures to EMF, crabs may be unable to repay this oxygen debt, potentially leading to increased mortality. Both D-Glucose and L-Lactate concentrations show high individual variability with D-Glucose levels influenced by individual status and reactions to external stimulus (Matsumasa and Murai, 2005). The values observed for L-Lactate and D-Glucose corresponded with those found in previous literature (Watt *et al.*, 1999; Lorenzon *et al.*, 2008; Barrento *et al.*, 2010; Barrento *et al.*, 2011). Haemocyanin, as the primary oxygen carrying protein in invertebrates, has been shown to increase in concentration during periods of hypoxia (Hagerman *et al.*, 1990). The lack of deviation in concentrations observed suggests that EMF exposure does not elicit similar physiological responses as hypoxic conditions. The overall lack of change on these parameters suggests this species can maintain homeostasis during exposure to high strength EMFs.

Although increased oxygen demand and high gill ventilation rates typically occur in crustaceans subjected to different stressors (Jouve-Duhamel and Truchot, 1985; Paterson and Spanoghe, 1997), EMF (2.8mT) did not significantly alter the respiration rate of juvenile crabs. Respiration rates in *Cancer pagurus* are highly variable due to the alternating periods of apnoea and bradycardia that have been observed in pausing behaviour (Bottoms, 1977; Burnett and Bridges, 1981). This pausing behaviour will alternate but can be present for significant periods of time. This was concluded by Burnett and Bridges (1981) when individuals were found to be exhibiting pausing behaviour for 40-50% of the time. These results show that juvenile *Cancer pagurus*

respiration levels correlate well with other species of crabs of similar size: velvet swimming crab, *Necora puber*, (0.21 ±0.119 mg O_2/g/h (Small *et al.*, 2010)); spider crab, *Hyas araneus*, (0.025 mg O_2/g/h (Camus *et al.*, 2002)); Dungeness crab, *Metacarcinus magister*, (0.044 mg O_2/g/h (Johansen *et al.*, 1970)) and shore crab, *Carcinus maenas*, (0.036 - 0.126 mg O_2/g/h (Newell *et al.*, 1972; Taylor and Wheatly, 1979). Current respiration values for adult *Cancer pagurus* found in the literature are 28.03 mg O_2/g/h during pre-pause and 4.42 mg O_2/g/h post pause (Bradford and Taylor, 1982).

The lack of deviation in the number of antennular flicks during initial exposure and throughout the trials suggest that the antennules may not be utilized in the detection of EMF in this species, or as a reliable indicator of detection. Similar results were reported by Woodruff *et al.* (2013) after exposing Dungeness crab, *Metacarcinus magister*, to a 3mT EMF.

Exposure to EMF did not have any effect on the overall activity level in *Cancer pagurus*. This suggests that if there is a behavioural change during exposure to EMF it may be more subtle than basic movement levels. The side selection results confirm that there is a decreased ability to find a suitable resting spot, however the crabs did not have higher activity levels within the EMF treatment. Under control conditions the crabs alternated their time between resting and roaming, subsequently spending larger amounts of time resting in the same spot. EMF exposure did not affect the resting and roaming behaviour but appeared to inhibit the crabs from spending large amounts of time in the same location. Overall activity levels were not affected by EMF exposure, but the distribution of time spent in specific locations within the tank and between resting and roaming behaviours were. The low activity levels during the day could be a result of behaviour consisting largely of shelter seeking (Chapman and Rice, 1971; Hockett and Kritzler, 1972; Hazlett and Rittschof, 1975; Hill, 1976). The discrepancies between size groups could be explained by smaller crabs typically inhabiting the sub-littoral zone where there will be higher risks of predation and higher competition for food and shelter, whereas larger crabs which tend to be found in deeper waters may not be subjected to the same pressures as the juveniles given their larger size (Paine, 1976). The increase in activity levels during the night corresponds with this species' nocturnal behaviour and will be due to foraging or potential mate seeking (Seed, 1969; Skajaa *et*

al., 1998). The increase in antennular flicking rate of larger crabs combined with the decreased activity levels suggest that adult crabs rely more on chemical sensing than physical exploration to survey the environment.

Exposure to EMF does not affect the activity levels of the crabs but affects their ability to select a site to rest. This may be explained by crabs seeking shelter when they detect EMF as opposed to natural movement patterns (Skajaa *et al.*, 1998) observed in those within the control group. The crabs under control conditions spent a higher percentage of their time on one side of the tank interspersed with active roaming. EMF exposure inhibited a clear side preference within the tank which resulted in an approximately 50-50% split across the tank, potentially reflecting shelter seeking behaviour. *Cancer pagurus* has been shown to inhabit pits when inactive (Hall *et al.*, 1991) and were observed spending large amounts of time resting during the day in acclimation tanks with minimal movement. This behaviour appears to have been altered by exposure to EMF.

During the single shelter trials when crabs were exposed to control conditions there was an equal amount of time spent inside and outside the shelters. The same pattern was recorded during the dual shelter trials, with an equal amount of time spent in either of the shelters and roaming the tank. This suggests that when there are no environmental stressors present the crabs will spend a portion of their time resting in shelter and an equal portion of their time surveying their environment exhibiting roaming behaviour. When there was an EMF present the amount of time spent exhibiting roaming behaviour significantly decreased in both single and dual shelter trials. This has clear implications on the *Cancer pagurus* population in the areas surrounding MREDs. If there is an EMF present, then crabs will be drawn to the source of the emission and spend significant amounts of time within the affected area. This will come at the cost of time spent foraging for food, seeking mates and finding shelter, potentially leading to higher predation rates, increased death due to starvation and/or decreased number of successful matings. Many offshore sites have introduced no-take zones around turbine arrays, with speculation that the decrease in fishing pressure, combined with the addition of artificial reefs in the form of scour protection blocks, could enhance the overall crustacean population (Langhamer and Wilhelmsson, 2009) by providing refuge and breeding areas. However, this experiment highlights the potential lack of spill-over effect from

these areas due to a high attraction to the emitted EMF. This suggests that fishing zones in close proximity to subsea power cables could potentially see an overall decrease in crab numbers. Scour protection zones are estimated to create 2.5 times more habitat than is lost by array installation (Linley *et al.,* 2009) and with the inclusion of drilled holes have an estimated carrying capacity of 65,000 kg of fish per year per turbine (Linley *et al.,* 2009). If specific habitat requirements are considered for individual target species around MREDs during the construction of these artificial habitats, then abundance and diversity of associated species, including commercially important species, could be enhanced (Bortone *et al.,* 1994; Kawasaki *et al.,* 2003) subject to EMF emission mitigation.

Several decapod crustaceans are known to be magneto sensitive, yet information available on the effects of electromagnetic fields emitted from MREDs is scarce. The aim of this study was to fill some of these knowledge gaps. Exposure to electromagnetic fields, of the strength predicted around sub-sea cables, had significant physiological effects on *Cancer pagurus* and changed their behaviour. EMF disrupted the circadian rhythm of haemolymph L-Lactate and D-Glucose levels. Melatonin levels in several species have been found to be affected by EMF exposure. This suggests that EMF exposure could affect crustaceans on a hormonal level. Further studies are needed to understand the underlying mechanism responsible for disrupted glucose and lactate cycles.

In this study it was shown that EMF exposure altered behaviour, with crabs spending less time roaming around the tank and more time in a shelter in direct contact with the EMF. This suggests that the natural roaming behaviour, where individuals will actively seek food and/or mates has been overridden by an attraction to the source of the EMF. When given the choice between a shelter exposed to EMF and one without exposure, the crabs were always drawn to the EMF. These results predict that in benthic areas surrounding MREDs, where there is increased EMFs, there will be an increase in the abundance of *Cancer pagurus* present. This potential aggregation of crabs around benthic cables and the subsequent physiological changes in L-Lactate and D-Glucose levels, brought about by EMF exposure, is a cause for concern.

Berried female edible crabs move offshore and spend 6-9 months buried with minimal movement and lower feeding rates (Williamson, 1900; Edwards, 1979; Howard, 1982; Naylor *et al.,* 1997). Given this species' proven attraction to EMF sources, incubation of the eggs may take place around areas with increased EMF emissions. Long term studies are needed to investigate the effects of chronic EMF exposure. The effects of EMF on egg development, hatching success and larval locomotory ability are unknown and need to be addressed. As larval stages are critical population bottlenecks, any negative effect of EMF on crab larvae will have a drastic effect on the edible crab fishery.

With the recent large-scale renewable energy developments, it is clear more research is needed to reduce uncertainty of the environmental effects of these activities on benthic marine species, particularly on other commercially and ecologically important decapod crustaceans. This is important to develop an understanding of population level consequences and cumulative impacts of MREDs' stressors. These knowledge gaps need to be addressed before the implementation of the many approved wind farm sites around the UK to help mitigate an ever growing problem.

Chapter 5. Behavioural and physiological responses in the European lobster, *Homarus gammarus* (L.), to electromagnetic field emissions from Marine Renewable Energy Devices (MREDs)

5.1 Introduction

In recent years there has been a significant increase in renewable energy, as a need to counteract the predicted decline of non-renewable energy in future decades (Pimentel *et al.*, 2002). Wind energy currently contributes around 15% of UK electricity generation (Department for Business, Energy & Industrial Strategy, 2018) and has one of the lowest levelized cost per MWh of electricity generation when a carbon cost is included in electricity generation (Department for Business, Energy & Industrial Strategy, 2016). Due to planning restrictions, lack of inexpensive land near population centres (Bilgili *et al.*, 2011) and aesthetic problems (Gill, 2005), renewable energy structures are increasingly being located offshore. The significant potential for generating energy offshore has led to an increase in offshore renewable infrastructure (Muller, 2013). The UK is perfectly situated to harness offshore wind and is currently the largest global producer of wind-derived energy, currently with more projects in planning or construction than any other country worldwide (The Crown Estates, 2016). Proposed sites are based on the current state of the art, which include the information provided from local environmental assessments and from scientific literature detailing suggestions on the effects of renewable energy on various habitats. However, significant gaps exist in the current knowledge of the effects of renewables on marine and freshwater organisms (Cada *et al.*, 2011; Scott *et al*., 2018a; Dannheim *et al.*, 2019). Previous studies suggest offshore renewables and their associated scour protection areas may enhance biodiversity through new habitat creation (Landers Jr *et al*., 2001; Lindeboom *et al*., 2011) whereas effects have also been found in fish (Lerchl *et al.*, 1998; Formicki *et al.*, 2004; Krzemieniewski *et al.*, 2004; Westerberg and Lagenfelt, 2008), crustaceans (Woodruff *et al.*, 2012; Scott *et al*., 2018a) and birds (Garthe and Huppop, 2004; Mendel *et al.*, 2019).

Marine Renewable Energy Devices (MREDs) are currently connected via sub-sea power cables, in addition to export cables from deployment site to shore. These sub-sea power cables, like most current carrying cables, emit electromagnetic fields (EMF). Currents of

around 850 to 1600 Amperes (A) (AC magnetic fields 715A (33kV cable) – 525A (220kV cable) (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019) are found in undersea power cables resulting in EMF emission of 3.2mT in a perfect wire (Bochert and Zettler, 2006). It has been shown in models that the strength of an EMF shortens the further it travels from the source cable (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019) , whereby 220kV cables produce a 22µT magnetic field at source that falls to 2µT 5m away from the cable. Similarly, a 33kV cable produces a 13µTesla magnetic field that falls to 0.5/1 µT 5m away from the source (Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019). Previous studies have shown that there is not currently an industry standard insulation that can successfully shield from both electric field (E-field) and magnetic field (B-field) emissions (Gill, 2005). The result of this B-field leakage combined with standard cable configurations results in an induced EMF (iEMF) (Gill, 2005) that could potentially cover large areas of the benthos around the deployment boundaries, depending on the scale and the number of cables used in a certain area. The resultant iEMF will be affected by saltwater ion movement via underwater currents near cables and the current type and strength passing through, which will result in an EMF of variable size and strengths that are extremely hard to predict. Normandeau *et al.* (2011) reported a great variation of EMF strengths around different structures associated with MREDs, whilst Thomsen *et al.* (2015) reported higher strengths around export cables, which in recent years utilise High Voltage Direct Current (HVDC) cables, rather than inter-device cables typically consisting of AC. A review of the literature (CMACS, 2003) indicates that the current knowledge on EMF strengths emitted from MREDs is insufficient to allow for an informed assessment. EMFs originate from both anthropogenic and natural sources: submarine telecommunication cables (Gill *et al.,* 2005), power cables, MREDs, and the Earth's natural magnetic field (Scott *et al.,* 2018a).

The European lobster, *Homarus gammarus*, is found throughout Western Europe from Norway to the Black Sea in the Mediterranean (Holthius, 1991). They are commonly found from immediate sublittoral to depths of around 60m (Templado *et al*., 2004). Typical habitats include rocky outcrops with sandy expanses, where food items consisting of benthic invertebrates, mainly molluscs, are present (Smith *et al*., 2001). They are a heavily exploited commercial species with 3,300 t, worth approximately £39.5million,

landed by UK fishermen in 2016 (Marine Management Organisation, 2016). Previous studies have shown that *H. gammarus* have been found to inhabit areas around marine renewable bases (Langhamer and Wilhelmsson, 2009). In studies by Leonhard and Pedersen (2006), Maar *et al.* (2009), Lindeboom *et al.* (2011), and Vandendriessche *et al.* (2014) it was concluded that new communities were established around scour protection zones at turbine bases of offshore wind farms. Scour protection zones established around wind turbine bases form artificial reefs by introducing hard substrate to areas that are typically sandy. These artificial reefs provide surfaces suitable for settlement of species including: worms, barnacles, and provide refuge for crabs and lobsters (Hunter and Sayer, 2009; Hiscock *et al.*, 2010). This highlights the increased probability that *H. gammarus* will come in to contact with sub-sea power cables resulting in increased EMF exposure.

The aims of the present study are to determine the effects of EMFs on *H. gammarus* using a combination of previously identified crustacean stress response parameters including: L-Lactate and D-Glucose haemolymph concentration (Taylor *et al.*, 1997; Durand *et al.*, 2000; Bergmann *et al.*, 2001; Scott *et al.*, 2018a, 2018b), Total Haemocyte Count (THC) (Durliat and Vranckx, 1983; Persson *et al.*, 1987; Smith *et al.*, 1995; Jussila *et al.*, 1997), attraction/avoidance, activity level (Stoner, 2012; Scott *et al.*, 2018a), and shelter selection (Scott *et al.*, 2018a). Haemolymph analysis is a common method for measuring stress in crustaceans (e.g. Hagerman, 1983,1986; Lorenzon *et al.*, 2011; Scott *et al.*, 2018a; Ooi *et al.*, 2019). L-Lactate (by-product of anaerobic respiration) and D-Glucose (fuel for ATP production in crustaceans) (Durand *et al.*, 2000; Lorenzon *et al.*, 2011) have been shown to follow circadian rhythms in another commercially important crustacean, the edible crab, *Cancer pagurus*, and have been proven to be affected by exposure to increased EMF (Scott *et al.*, 2018a). Haemocytes play an important role in the immune responses of crustaceans via phagocytosis and encapsulation of foreign bodies (Johansson *et al.*, 2000). A study by Perazzolo *et al.* (2002) showed that the Carpas shrimp, *Farfantepenaeus paulensis*, expressed a 40% reduction in THCs during exposure to lower salinities. Activity level of decapods is a useful tool to measure stress as they have been shown to exhibit an avoidance response to stressors in the marine environment (Filiciotto *et al.*, 2014; Smyth *et al.*, 2014). Previous studies by Smyth *et al.* (2014) highlighted avoidance behaviour in *H. gammarus* and *C. pagurus* when exposed to altered salinities

and Scott *et al.* (2018a) showed that *C. pagurus* showed a change in attraction/avoidance behaviour and a defined change in shelter selection when subjected to an artificial EMF.

5.2 Materials and Methods

A total of 161 intermoult lobsters (83 female and 78 male) were collected from local fisherman in the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK). Lobsters were kept in 1000 L holding tanks (at densities of 4 lobsters/tank) which maintained ambient sea temperature through a constant supply of filtered and U.V. sterilised seawater for a week prior to experimentation. Due to the nature of the animals, holding tank densities remained at a maximum of 4 lobsters to prevent and limit physical damage due to aggression. Holding tanks and experimental tanks were subjected to a natural photoperiod via a transparent laboratory roof. Lobsters were held in holding tanks for an acclimation period of 1 week. Lobsters were sexed, weighed (g) and carapace measured (mm) before use. Lobsters were subjected to a condition index (Scott *et al.*, 2018a) whereby, lobsters with a maximum of two chelipod/periopods missing and or showed no signs of carapace damage were used for the trials. Throughout all experiments, an approximate equal split of male (N=21 physiological analysis, N=57 behavioural analysis) and female (N=12 physiological analysis, N=71 behavioural analysis) lobsters was used to detect any potential variances, between sexes, in response to treatments. Experimentation was conducted July – August 2018.

Helmholtz Coil

Two $2m^3$ Helmholtz coils (one control and one EMF) were set up with six 19 L glass tanks supplied with temperature controlled (14±0.5°C), UV sterilised, filtered seawater at a flowrate of 0.5 l/min. Tanks were placed in black plastic storage boxes acting as both water baths and as a visual barrier, reducing external visual stimuli. Experimental tanks were placed within the coils where EMFs are homogenous to approximately ±5%. Temperature, dissolved oxygen, salinity, and light intensity were constantly monitored via data loggers (Onset HOBO temperature/light pendant). EMF within tanks were

mapped using an Alpha Lab, Inc. Gaussmeter Model GM-2. EMF strength of 2.8mT was obtained to follow those utilised in previous studies (Bochert and Zettler, 2006; Scott *et al.*, 2018a) and was uniformly present throughout experimental area. The Helmholtz coil was powered using a variable DC power supply (Elektro-Automatik EA-PSI 8360-15).

Physiological analysis

Haemolymph analysis

To test the effect of EMF on lobster haemolymph parameters 17 (10 male and 7 female) lobsters were exposed to a static DC EMF (2.8 mT) and 17 (11 male and 6 female) lobsters were kept under control conditions (coils not powered) for a 24 h period. Similar size intermoult lobsters were used in trials with a mean weight of 500.8±192 g and carapace length of 90.2±6 mm. Prior to testing, lobsters were fed three times a week with cooked mussel and fish, with feeding discontinued 48h before the trials. To ensure natural conditions, tanks were enriched with a thin layer of sediment consisting of sterilised coarse shell sand and small pebbles. Within each tank one shelter (ABS pipe, l=200 mm, d=100 mm) was placed to provide refuge. Tanks were aerated with a single air stone and supplied with flow-through UV sterilised, temperature controlled (TECO TK1000, 14 °C), filtered sea water. Lobsters (one per tank) were acclimated in the experimental tanks for 24 h prior to experimentation. Temperature (14±0.1°C), dissolved oxygen (>90%), salinity (34±0.5 ppt) were monitored and kept constant throughout trials. A clear aquarium roof allowed a natural photoperiod of approximately 15 h light-9 h dark. Tanks were drained, cleaned and sterilised (Virkon aquatic) after each trial Haemolymph samples were collected at 0 h (09:00am), 6 h (15:00pm), 12 h (21:00pm) and 24 h (09:00am). Sampling times were chosen to determine if a circadian rhythm of D-Glucose and L-Lactate was present over a 24 h period. It has previously been documented in other decapods that D-Glucose levels increase during evening periods of higher activity (Gorgels-Kallen and Voorter., 1985; Kallen., 1990). Haemolymph (700 µl) was collected from the pericardial sinus (post cleaning using 70% ethanol) using 1ml syringes with 25G needles which had been stored at -25°C prior to sampling. Sampling did not exceed 60s to reduce stress to the animals and help prevent coagulation. From the haemolymph samples: 300 µl was used for D-Glucose assay, 300 µl for L-Lactate assay, and 100 µl for Total Haemocyte Counts. The extracted haemolymph was transferred to 1.5 ml cryogenic

vials (Nalgene) and then stored on ice (Total Haemocyte Count) or the freezer (D-Glucose and L-Lactate tests) prior to analysis.

Haemolymph was deproteinised for D-Glucose and L-Lactate assays, as per Paterson and Spanoghe (1997). 600 µl of 0.6 M perchloric acid (BDH 10175) was added to 600 µl haemolymph then centrifuged (Eppendorf 5417C, rotor 30 x 1.5-2 ml) at 25,000g for 10 min to separate the inactive proteins. The supernatant was extracted and neutralised with 50 µl of 3 M potassium hydroxide (BDH 29628). 500 µl of the supernatant was transferred to a new vial and stored in a freezer at -25°C. No significant differences were found between male and females during preliminary analysis, as such the data was pooled together for subsequent analysis.

D-Glucose

D-Glucose concentration was determined using the D-Glucose assay kit (GAGO20-1KT) as per Barrento *et al*. (2010). The stored haemolymph was thawed before analysis, 150 µl of the sample was mixed with 300 µl reagent assay and incubated for 30 min at 37°C in a water bath. The reaction was stopped using 300 µl of 12 N sulphuric acid (BDH). Absorbance was then measured in parallel measurements in microcuvettes at 540nm. D-Glucose concentrations were then calculated using a calibration curve of standards with a known concentration.

L-Lactate

Deproteinated haemolymph samples were analysed for L-Lactate concentration using colorimetric L-Lactate assay kit (Abcam ab65331). 50 µl of reaction mix (L-Lactate assay buffer (46 µl), L-Lactate substrate mix (2 µl) and L-Lactate enzyme mix (2 µl)), were added to a 50 µl deproteinated haemolymph sample. The reaction mix and sample mix were then incubated at room temperature for 30 min then spectrophotometrically analysed in parallel measurements at 450nm. Concentrations were determined using a curve of values produced by spectrophotometrically assessing calibration standards of known concentrations.

Total Haemocyte Count

50 µl haemolymph was drawn from the pericardial sinus with sterile pre-chilled 1ml syringes (25 gauge) containing 150 µl cooled 5% (v/v) Formaldehyde (Brunel Microscope Ltd.). Haemolymph samples were dispensed to centrifuge tubes, mixed thoroughly and kept on ice to prevent coagulation. Total Haemocyte Count (THC) of individual lobsters were estimated with a Neubauer haemocytometer under magnification (X100) with a Leica (MC170 HD) compound microscope. For accuracy, samples were counted in triplicates. THC was expressed as number of cells in 1 ml of haemolymph.

Behavioural analysis

Activity level and side selection

Two 70 L experimental tanks were connected to a 1000 L temperature-controlled (14°C) sump tank which received a constant supply of UV-sterilised filtered sea water. Four solenoid lifting electromagnets (12V) were connected to variable DC power supplies (QW-MS305D) and placed beneath the experimental tanks resulting in half of the tanks being exposed to an EMF of 2.8mT as per Scott *et al.* (2018a). During the trials one tank would have the magnets switched on with the other remaining off as the control. The tanks acting as the control were swapped between trials to minimise tank effect. Dark fine-mesh netting was placed under each tank to set a neutral non-biased background to prevent visual stimuli from affecting the trials. Tanks were drained, cleaned and sterilised (Virkon aquatic) after each trial. Dissolved oxygen (>90%) and salinity (34±0.5 ppt) in experimental tanks were monitored and kept constant. Waterproof infrared cameras (Sannce 1080p IR surveillance DVR system) were suspended above each tank and set to record from 23:00pm to 06:00am which coincides with the period of highest activity recorded in *H. americanus* as described by Golet *et al.* (2006). The footage was then post processed (FormatFactory V4.3.0) and analysed using Solomon Coder (beta version 17.03.22). Each video file was broken down to still images at 1-min intervals. These still images were used to assess the activity level and side preference throughout trials. Movement index was categorised into movement (denoted by a 1) or no movement (denoted by a 0) based on the previous image, whilst side preference was categorised by magnet (M) or non-magnet (U) side. The activity level was determined as the cumulative number of 1s throughout the trial analysis expressed as a percentage. Total movement

index score of the lobster, over the 7 h period, indicated the activity levels of the animals whilst the percentage of time spent on each side indicated an attraction or avoidance to EMF. The EMF was mapped across the base of the tank using 1sq. inch grids measured using an AlphaLab, Inc. Gaussmeter Model GM-2. A total of 37 lobsters were used for side selection (18 EMF and 19 control) and 91 for activity level (36 EMF and 45 control). One lobster was used per tank with different individuals used for side selection and activity level experiments.

Shelter selection

Four 70 L tanks were set up as in the previous side selection study, with a constant supply of temperature controlled (14°C) flow-through UV sterilised and filtered seawater. Tanks contained either one (single EMF) or two (dual EMF) black ABS plastic shelter(s) (300 mm x 200 mm x 100 mm) secured to the base of the tank (Figure 5.1.). Four solenoid electromagnets were placed under all shelters, during single shelter trials one set of magnets were turned on with the other acting as a control. For the dual shelter trials, one shelter in each tank had the magnets switched on creating an EMF (2.8mT) throughout the shelter. Individual lobsters were placed in each tank, maintaining an approximate even split of male and females. A total of 15 lobsters were used in both treatments (Control and EMF). Using the same infrared camera set-up previously described, the lobsters were recorded from 23:00pm – 06:00am and the videos post processed and analysed using Solomon Coder (beta version 17.03.22) and FormatFactory (V4.3.0). The analysis involved assessing the presence or absence of the lobster within the shelter (single shelter set-ups) or shelters (dual shelter set-ups) and the time spent out with the shelters. The method followed the activity level assessment as described in the previous section.

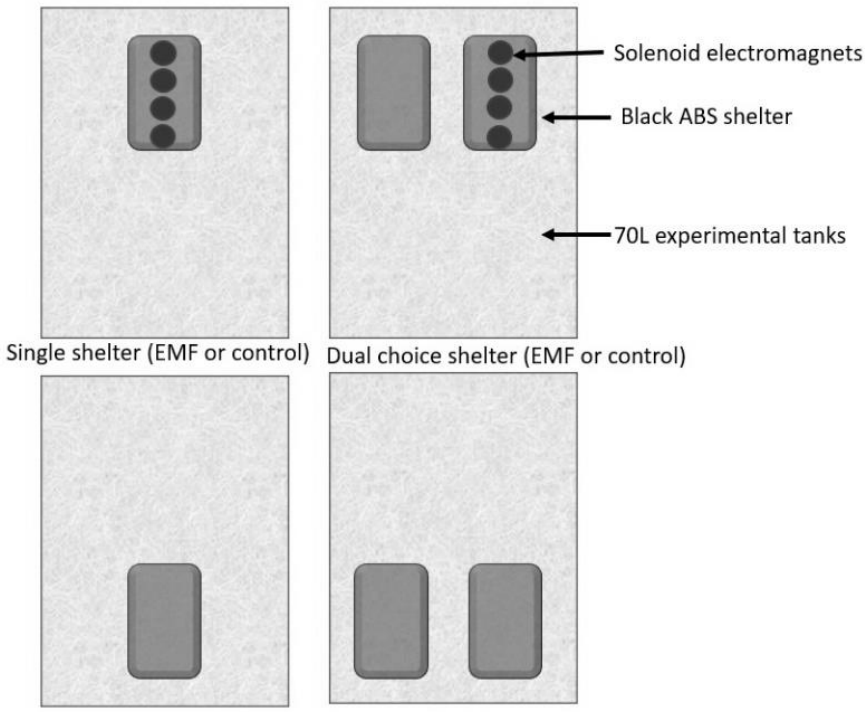


Figure 5.1. Experimental set-up of the shelter selection tanks, as per Chapter 4. Four 70 L experimental tanks with four solenoid magnets placed underneath which were switched on to emit a 2.8mT electromagnetic field.

Statistical analysis

Results were expressed as mean value ± standard error (SEM). When data met ANOVA assumptions (per Shapiro-Wilk test for normality and Levene's test for equality of error variances) multiple comparison tests (paired t-test, one-way ANOVA, 2-way ANOVA) were conducted to reveal differences between groups. If data could not meet ANOVA assumptions, non-parametrical analyses (Wilcoxon signed rank test, Mann-Whitney, Scheirer-Ray-Hare) were performed. Choice experiments were tested using chi-square test (2 tailed) with Yates correction. Post-hoc analysis for parametric data (Tukey's test) and non-parametric (pairwise Mann-Whitney) were conducted. All statistics were tested at a probability of 0.05 (IBM SPSS Statistics v.23 SPSS Corp. Chicago, USA).

5.3 Results

Physiological analysis

Haemolymph analysis

L-Lactate concentrations of *H. gammarus* were significantly affected by exposure to EMF (Figure 5.3.). At 12 h of exposure the mean haemolymph L-Lactate concentration was significantly higher than the original 0 h sample ($t(9)=-2.95$, $p<0.05$, paired t-test). L-Lactate concentrations at 6 h and 24 h were higher than the original sample at 0 h however this was not statistically significant. Under control conditions there were no significant variations in L-Lactate concentration throughout 24 h. D-Glucose concentrations showed significant increases between 0h and all subsequent samples in EMF exposed lobsters ($t(10)=-3.46$, $p<0.01$, paired t-test) (Figure 5.2.). Under control conditions there were no significant differences between mean D-Glucose concentrations for all sample times despite showing continual increases.

Under control conditions there were no significant differences in THC between sampling times, with values averaging between $41.2\pm5.2 \times 10^6$ and $46.2\pm5.7 \times 10^6$ cells/ml. The total number of Haemocytes in lobsters exposed to EMF showed a slight decrease after 6h ($40.3\pm5.7 \times 10^6$ cells/ml) and significantly lower values after 12 h of exposure ($37.5\pm5 \times 10^6$ cells/ml) compared to the control group ($t(32)=2.32$, $p<0.05$, t-test). After 24 h of EMF exposure THC values were significantly elevated ($t(11)=-3.40$, $p<0.01$, paired t-test) compared to THC after 6 h exposure but showed no significant difference from the 0 h ($42.72\pm5.8 \times 10^6$ cells/ml) and control 6 h ($46.5\pm6.2 \times 10^6$ cells/ml).

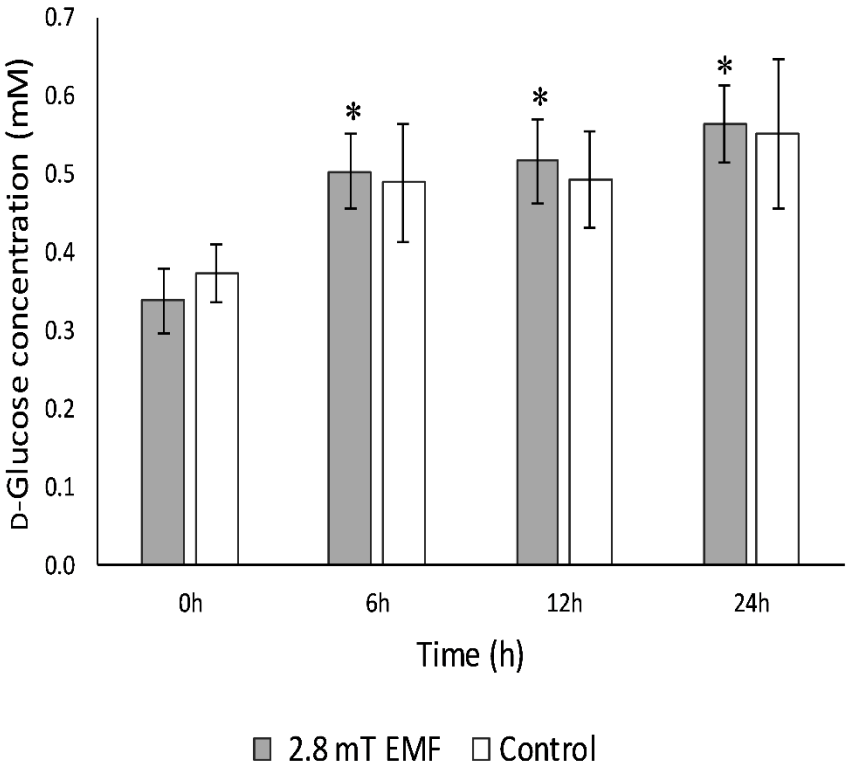


Figure 5.2. D-Glucose concentrations over a 24 h period under control and EMF exposed (2.8mT) conditions. D-Glucose concentrations did not seem to follow a circadian rhythm and remained relatively constant throughout the 24 h period under control conditions. Exposure to EMF showed significantly elevated values at every time point compared to 0 h ($p<0.05$). Values are presented as Mean ± SEM, * is the significance from 0 h ($p<0.05$). N=34 (21 male and 13 female) (17 EMF 17 control).

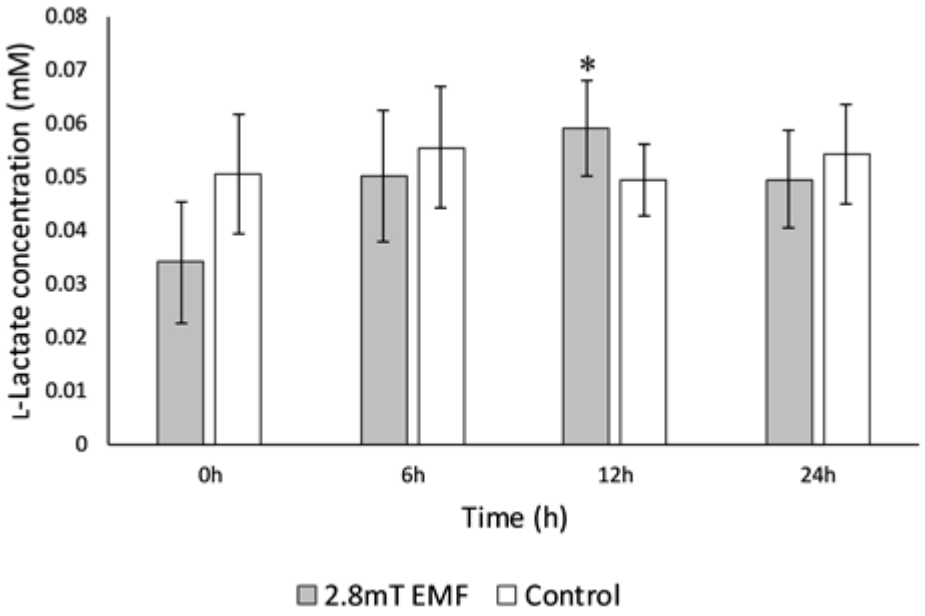


Figure 5.3. L-Lactate concentrations over a 24 h period during exposure to 2.8mT EMF and control conditions. No circadian rhythm was noted in L-Lactate under control or EMF exposed conditions. Exposure to EMF (2.8mT) resulted in a significantly higher concentration of L-Lactate after a 12 h exposure when compared to 0 h. Values are presented as Mean ± SEM, * is the significance from 0h ($p<0.05$). N=34 (21 male and 13 female) (17 EMF 17 control).

Behavioural analysis

Activity level

Exposure to EMF had no significant effect on the activity levels of *H. gammarus*. Under control conditions lobsters were active for 36.7% of the trial (7h) whilst during EMF exposure lobsters were active for 33%. There were no significant differences in activity levels between male and female lobsters.

Side selection

Under control conditions lobsters spent significantly more time on one side of the tank (64.4% - 35.6%) ($p<0.01$, Chi square test). During exposure to EMF the mean time spent on either side of the tanks remained similar to those found under control conditions (68.7% - 31.3%) with a significant preference for one side of the tank ($p<0.01$, Chi square

test). However, as tank configurations were rotated and randomised to avoid tank effect, there were no clear preferences for either the side with the magnets nor the control side in any trials.

Single shelter preference

Mean time spent in the shelter was significantly higher in both EMF (382.33±17.54 min) and control (403.4±5.49 min) conditions than time spent out roaming the tank (37.67±17.54 min and 16.6±5.49 min for EMF and control respectively, one-way ANOVA $F(1,36)=783.3$, $p<0.001$) (Figure 5.4.). Exposure to EMF had no significant effect on shelter utilisation and subsequent time spent roaming the tank throughout the single shelter trials.

Dual shelter preference

Under control conditions the mean time spent in either shelter was approximately equal (17.33±5.9 min and 10.2±4.2 min for control shelter and EMF shelter respectively), with a small portion of time spent roaming the tank (4%). During exposure to EMF there was a clear preference for the shelter at the source of the EMF resulting in a mean time of 337±29.5 min spent within, with 76.3±29 min spent in the control shelter and 10.2±4.2 min spent roaming the tank (Figure 5.4). There was a 19% decrease in the mean time spent within the control shelter and a subsequent increase of 13% mean time spent within the EMF shelter, when compared to control trials. The overall mean time spent in all three locations were significantly different between control and EMF conditions ($F(1,51)=6.4$, $p<0.001$, one-way ANOVA).

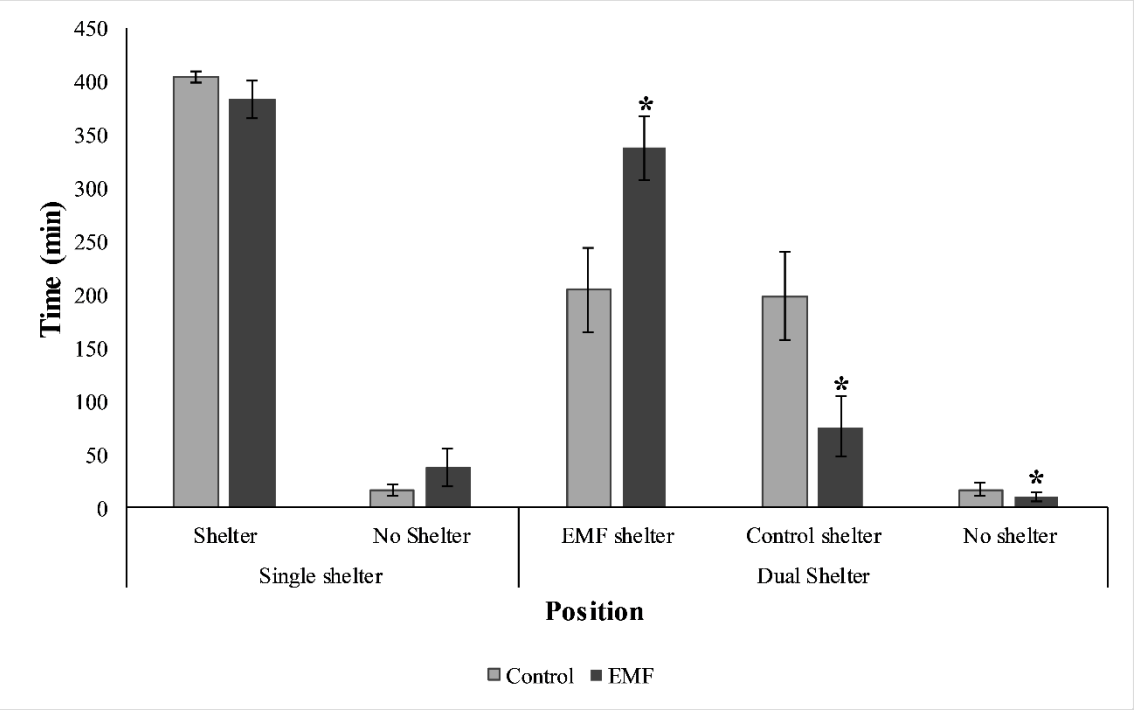


Figure 5.4. The effects of EMF exposure on shelter selection in *H. gammarus*. Mean time spent in the shelter and roaming the tank in both control and exposure to 2.8mT EMF in single shelter trials. Mean time spent within control and EMF exposed (2.8mT) shelter and roaming the tank in dual shelter trials. Calculated as time spent in each location compared to total trial length. Values are presented as Mean ± SEM, * is the significance $p<0.05$ compared to the control group. Single shelter $N_{CONTROL} = 17$, $N_{EMF} = 21$, dual shelter $N_{CONTROL} = 28$, $N_{EMF} = 25$.

5.4 Discussion

L-Lactate and D-Glucose concentrations in *H. gammarus* do not appear to follow a natural circadian rhythm with subsequent peaks and troughs as has been found in *C. pagurus*, another commercially important decapod found throughout Western Europe (Scott *et al*., 2018a). These cycles, which were found to result in a rise in D-Glucose throughout the day and a subsequent fall in L-Lactate and the opposite occurring at night, may still be present in *H. gammarus* but may take place over a longer period of time, and were not detected during the sampling times utilised throughout this experiment. Previous studies have found that environmental stressors influence glucose and lactate concentrations in crustacean haemolymph (Kallen *et al.*, 1990; Reddy *et al.*, 1996; Chang *et al.*, 1998; Scott

et al., 2018a; Harrington and Hamlin., 2019). D-Glucose is the primary fuel for ATP production in crustaceans and is essential in maintaining metabolic processes (Barrento *et al.*, 2010), as such a negative relationship exists with moribund crustaceans becoming hyperglycaemic, and subsequently lower values found in healthy individuals (Barrento *et al.,* 2010). The D-Glucose values obtained throughout these experiments correlated well with those found in the literature. A study by Radford *et al.* (2005) found that baseline haemolymph glucose concentrations of juvenile rock lobsters, *Jasus edwardsii*, was 0.61±0.02 mmol l^{-1}. Spanoghe and Bourne (1999) reported mean D-Glucose concentrations ranging from 0.16±0.05 to 1.25±1.12 mmol l^{-1} in spiny lobster, *Palinurus Cygnus.* The American lobster, *Homarus americanus,* was found to have a mean range of 0.68±0.19 to 2.04±0.83 mmol l^{-1} D-Glucose concentration, dependent on the body temperature of the individual (Lorenzon *et al.,* 2007). D-Glucose values for other crustaceans fall in to a similar range including: 0.09 to 1.40 mmol l^{-1} in *C. pagurus* (Scott *et al.,* 2018b), 0.77 to 1.39 mmol l^{-1} in giant tiger prawn, *Panaeus monodon,* (Hall and Van Ham, 1998), 0.9±0.2 mmol l^{-1} in Louisiana crawfish, *Procambarus clarkia,* (Garcia *et al.,* 1993), and 0.49 mmol l^{-1} in Jonah crab, *Cancer borealis,* (Glowik *et al.,* 1997).

Exposure to 2.8mT EMF caused significant increases in D-Glucose concentrations over a 24 h period, suggesting the onset of hyperglycaemia and subsequently confirming a state of increased physiological stress. Balanced D-Glucose concentrations are paramount to the function of certain organs, namely brain and muscle, and are strictly regulated both quantitively and temporarily (Verri *et al.,* 2001). This research shows that when subjected to EMF's D-Glucose concentrations steadily increase over the course of 24 h showing significant increase compared to control lobsters, which may lead to a significant decrease in vigour (Barrento *et al.,* 2010), an inability to produce ATP at a sufficient rate (Barrento *et al.,* 2010), and have a detrimental effect on brain and muscle function. D-Glucose concentrations are linked with activity levels in *C. pagurus* under normal unstressed conditions (Briffa and Elwood, 2001) and this was confirmed for *H. gammarus* during these experiments, where D-Glucose concentrations showed continued elevation throughout the day, with higher values obtained around dawn and dusk when activity levels for this species increased (Mehrtens *et al.,* 2005). A similar trend was also found within the control animals. Spinycheek crayfish, *Orcotnectes limosus,* exhibit a physiological increase in D-Glucose in the first 3 to 4 hours post-dusk (Kallen, 1990), whereas, Narrow-clawed crayfish, *Astacus leptodactylus,* show a constant increase

throughout the night (Gorgels-Kallen and Voorter., 1985). This shows that the increase in D-Glucose during the evening varies between decapod species. As *H. gammarus* is more active during evening hours it is consistent that an increase in D-Glucose concentration should occur in the build up to this increase in activity level (Scott *et al.*, 2018a). In the literature, D-Glucose concentrations have been found to increase with an increase in locomotor activity (Hamann, 1974; Reddy *et al.,* 1981; Kallen *et al.,* 1988; Kallen *et al.*, 1990; Tilden *et al.*, 2001; Scott *et al.,* 2018a).

L-Lactate, an indicator of anaerobic respiration, is typically due to reduced gill function in crustaceans (Durand *et al.,* 2000). A significant increase in L-Lactate in lobster haemolymph occurred after 12 h of EMF exposure. This suggests a higher degree of anaerobic respiration occurs after the onset of EMF exposure, with concentrations returning to those comparable with control after 24 h. Increased L-Lactate has been associated with increasing the O_2 affinity of Haemocyanin (Sanders and Childress, 1992) to aid in periods of hypoxia. In shore crab, *Carcinus maenas,* L-Lactate concentrations significantly rose during periods of emersion and remained elevated for 1 h after immersion which suggests the need for the organism to repay an oxygen debt (Simonik and Henry, 2014). This oxygen debt could partially explain the extended response in *H . gammarus* after EMF exposure and the subsequent decrease to baseline concentrations after 12 h. Previous studies on the effects of EMF exposure on *C. pagurus* found that L-Lactate concentrations significantly decreased throughout exposure, potentially reducing the ability of this species to adapt and recover from periods of hypoxia and/or emersion (Scott *et al.,* 2018a). This highlights a difference in response to EMF on L-Lactate concentrations of two species that cohabit areas around MREDs. L-Lactate concentrations were highly variable between individuals, although correlated well with those found in the literature for the closely related *H. americanus* (0.20±0.02 mmol l^{-1}) (Rose *et al*., 1998) although were far lower than those recorded for resting *H. gammarus* (0.9 mmol $^{l-1}$) by Taylor and Whiteley, (1989). This may be due in part to the increased sensitivities of modern assay kits, different means of obtaining haemolymph, and different methods of determining L-Lactate concentrations. Melatonin, a neuropeptide, has been linked to the regulation of L-Lactate and D-Glucose cycles in crustaceans (Tilden *et al.*, 2001). EMF exposure has been shown to influence melatonin levels (Levine *et al.*, 1995; Wood *et al.*, 1998) which suggests that the deviations within L-Lactate and D-Glucose found

throughout this experiment may be as a result of melatonin levels being directly altered during EMF exposure.

Haemocytes in crustaceans are known to contain potent antibacterial compounds that work against both gram-positive and gram-negative bacteria and have been found to work in low concentrations (Chrisholm and Smith, 1992). Lobster THC at 12 h was significantly lower in the EMF exposed lobsters than in control. THC counts decline in the presence of pathogenic bacteria, and or during starvation in *H. americanus* (Stewart, 1967). Le Moullac *et al.* (1998) also found a decrease in THC within western blue shrimp, *Litopenaeus stylirostris,* after exposure to severe hypoxia. Previous studies have also found that EMF exposure reduced THC of cabbage looper, *Trichoplusia ni,* larvae after 48 h exposure (Valadez-Lira *et al.,* 2017). The reduction in THC during EMF exposure suggests an impaired immune system and consequently a decreased ability to fend off bacterial attacks.

In EMF exposed lobsters there was a significant increase in THC between 6h and 24h which was absent in the control. An increase in THC typically indicates an increased immune response in crustaceans (Le Moullac and Haffner, 2000). In striped venus clam, *Chamelea gallina,* THC levels would significantly rise during exposure to high temperatures (Monari *et al.,* 2007). To our knowledge this is the first time that the effects of EMF exposure on crustacean THC has been assessed. As haematocytes have a crucial role in the immune defense of crustaceans, there is a concern that EMF could potentially affect European lobster immune-physiological responses. The lower concentrations detected in EMF exposed lobsters at 12 h, when compared to the control, suggests an impaired immune system resulting in a decreased ability to defend against pathogenic bacteria. The lower concentrations appeared to be temporary (restricted to the first 12 h of exposure), with an immunological response resulting in the significant increase in Haemocytes from 6 h to 24 h which suggests an increase in Haemocyte production to combat the stress of EMF exposure. The results appear to show that exposure to increased EMF causes an immune stress response in *H. gammarus*.

Activity levels remained unchanged throughout exposure to EMF, suggesting that if behaviour is affected by EMF it may be on a subtler level than basic movement patterns, however this would require further investigation. Activity levels found in lobsters (*H. americanus* and *H. gammarus*) reflect a crepuscular behaviour with increased activity

around dawn and dusk (Reynolds and Casterlin, 1979; Lawton, 1987; Mehrtens *et al.*, 2005; Golet *et al.,* 2006). Activity level experiments were conducted from 23:00pm – 06:00am (incorporating natural light cycles) to incorporate these periods of highest activity levels, assuming *H. gammarus* adopts a similar endogenous circadian rhythm. Although previous studies have found there is a high degree of variation in movement in lobsters (Golet *et al.,* 2006) there was little variation found throughout this experiment. Activity levels and movement of lobsters has been previously studied under laboratory conditions and under natural or semi-natural conditions with the conclusion being that there is little variation in behaviour (Golet *et al.,* 2006). There were no significant differences in the activity levels between male and female lobsters, which correlates well with previous research on *H. americanus* (Golet *et al.,* 2006).

H. gammarus did not exhibit an attraction to EMF based solely on side selection. In a similar study on *C. pagurus* a clear side preference existed under control conditions but was absent during exposure to EMF (Scott *et al.,* 2018a). Similar results were obtained throughout this experiment with lobsters showing a clear side preference within the tank. However, no change was noted in this behaviour during exposure to EMF. Throughout the trials the experimental tanks were rotated to limit tank effect, which suggests that side preference may have been chosen randomly and subsequently reflects resting periods between periods of increased activity. It was noted during these trials that lobsters spent greater periods of time resting in their chosen area of the tank at the beginning of the experiment and towards the end with increased movement occurring at the times in between. This is further confirmed in studies which have found a link between the decrease in light levels and the subsequent increase in decapod activity (Cooper and Uzmann, 1980; Lawton, 1987; Wahle, 1992; van der Meeren, 1997; Skajaa *et al.,* 1998; Golet *et al.,* 2006).

Single shelter trials were designed to determine if lobsters will choose the only available shelter despite being subjected to EMF. During these trials it was determined that whilst EMF exposure had no effect, lobsters utilised the shelter for over 90% of the trial time under both conditions, despite trial times coinciding with predicted peak locomotor activity (Golet *et al.,* 2006). Shelter availability is suspected to be a significantly limiting factor for lobsters in benthic environments (Caddy, 1986; Fogarty and Idoine, 1986). Aside from lobsters, other benthic crustaceans are also shelter-dependent including

hermit crabs (Bertness, 1981), crayfish (Stein and Magnuson, 1976) and spiny lobsters (Yoshimura and Yamakawa, 1988). The observed amount of time spent utilising the shelter during single shelter trials suggests that lobsters will prioritise shelter seeking behaviour over natural roaming and exploration behaviour due to the apparent bottleneck of shelter availability in the wild (Wahle and Steneck, 1991). Van der Meeren (2002) found that those *H. gammarus* exposed to a touch prior to experimentation, expressed a quicker time to reach the shelter compared to those without prior touching, suggesting stress will impact shelter seeking behaviour. When two shelters were present the mean time spent in each over the course of the trials was approximately equal with a small proportion of time spent roaming the tank. These results correlate well with those found in the single shelter experiments and continue to highlight the significant amount of time spent within a shelter compared to outside roaming the tank. When given a choice between an EMF exposed shelter and a control shelter there was a clear preference for the EMF shelter with a decrease in the mean time spent in control. Although contradictory to the initial results obtained throughout the single shelter experiments, this suggests that lobsters can detect EMF and show some form of attraction to it. Previous studies have shown that fish show an increased curiosity reflex when an EMF is present resulting in a higher catch with magnet equipped nets (Formicki *et al.,* 2004). An increased curiosity reflex may be partially responsible for the results obtained during shelter selection trials in this study. For example, as shelter availability tends to be a limiting factor in benthic lobster species, when presented with one shelter lobsters would occupy it for large periods of time, regardless of exposure to EMF. This suggests that shelter seeking behaviour may override potential effects of EMF exposure on the lobsters. When presented with two shelters, the pressure to seek and occupy a shelter will have decreased and perhaps enabled lobsters to select based on preference as opposed to necessity. The attraction to EMF was also found in the edible crab, *C. pagurus* (Scott *et al.,* 2018a) during similar shelter trials. Scour protection zones set up around the base of MREDs to reduce erosion, also act as artificial reefs and subsequently increase habitat by as much as 2.5 times than that lost by array installation (Linley *et al.,* 2009). These factors combined with the many no-take zones around turbine arrays will enhance overall crustacean populations (Langhamer and Wilhelmsson, 2009) with studies by Krone *et al.* (2013, 2017) showing that double the *C. pagurus* numbers are present on turbines with scour protection zones, 27% of the local *C. pagurus* stock will come from these areas and that a predicted 60 –

165% increase in substrata-limited mobile demersal species will occur in these areas. Both *H. gammarus* and *C. pagurus* have been shown to inhabit these artificial reefs (Langhamer and Wilhelmsson, 2009), however, this experiment suggests that there may be a lack of spill-over effect to the surrounding area due to the high attraction to the emitted EMF. In a study by Kawasaki *et al.* (2003) it was suggested that if the habitat requirements for specific target species were considered during MRED scour zone and artificial reef construction then the abundance and diversity of associated species could be enhanced. In addition to habitat creation, cable configuration, cable protection and positioning would also need to be assessed to mitigate EMF exposure affecting the target species attracted to these areas in either negative, neutral or perceived beneficial ways.

Exposure to EMF, of the strengths predicted around sub-sea cables for MREDs, has a significant effect on the behaviour of this species. Shelter seeking behaviour in lobsters is prominent and when given the choice between an EMF exposed shelter and control, a clear attraction was noted for the shelter at the source of the EMF. This has clear implications for this species around MREDs and suggests that within these areas there may be an increased abundance of *H. gammarus*, due to this attraction. The advent of creating artificial reefs from scour protection zones around turbine bases has been proven to increase biodiversity (Landers Jr *et al*., 2001; Lindeboom *et al*., 2011) and the presence of *H. gammarus* around these areas has been confirmed (Langhamer and Wilhelmsson, 2009). However, the holding capacity of these areas may not be large enough to sustain the predicted influx of lobsters that are drawn by a strict attraction to the underlying EMF emissions. This may result in higher mortality due to lack of suitable shelter and subsequent increase in predation of vulnerable individuals. The physiological changes that were determined during EMF exposure, such as elevated D-Glucose and L-Lactate concentrations, suggest that melatonin levels, which have been linked with the circadian rhythms of both D-Glucose and L-Lactate (Tilden *et al.*, 2001), could be affected by increased EMF resulting in higher concentrations. The combined physiological effects, suggest that lobsters have increased anaerobic respiration, begin to show signs of hyperglycaemia, and develop signs of an immunological response through THC variations, during exposure to EMF. The aggregations of lobsters predicted around these areas and the subsequent detrimental physiological changes noted in this study are a concern and future research is needed on the effects of chronic EMF exposure on haemolymph stress parameters.

Several decapod crustaceans are known to be sensitive to EMF (Boles *et al.*, 2003; Scott *et al.*, 2018a), however information in the literature is scarce on the impacts of MREDs on these species. *C. pagurus*, which shares a similar geographical area and habitat, has been shown to be negatively affected by EMF exposure on both a physiological and behavioural level and this study aimed to determine if the same were true for *H. gammarus*. Further research is needed to fully determine the effects of EMF exposure on all life stages of crustaceans. With the rapid increase in MREDs worldwide there is a need for further research to obtain a clearer understanding and attempt to mitigate potential impacts that may arise in the operational phase of these structures.

Chapter 6. The effects of electromagnetic field (EMF) emissions from Marine Renewable Energy Devices (MREDs) on egg development and larval locomotory ability of two commercially important crustaceans, edible crab, *Cancer pagurus* (L.), and European lobster, *Homarus gammarus* (L.)

6.1 Introduction

Electromagnetic fields (EMF) are a potential stressor associated with all marine renewable energy devices (MREDs), that is becoming increasingly prevalent (Pimental *et al.,* 2002; Scott *et al.,* 2018a). EMFs originate from a variety of both anthropogenic (sub-sea power cables, telecommunication cables) and natural sources (Earth's geomagnetic field) (Scott *et al.,* 2018a). MREDs, particularly wind farms, are interconnected via cable arrays through which, after conversion from AC to DC, electricity is exported to shore via High Voltage Direct Current (HVDC) export cables. These subsea power cables, like all current carrying cables, will emit EMFs of varying strengths. Currently, there is no industry standard insulation that can prevent magnetic field (B-field) leakage, whereas standard electric fields (E-field) are eliminated (Gill, 2005). Consequently, B-fields leaking from individual cables interact with each other, creating an induced EMF (iEMF) which will vary in strength depending on current strength in cables, water current speed and proximities of associated cables. (Bochert and Zettler, 2006; Normandeau *et al.,* 2011; Scott *et al.,* 2018a).

Currents between 850 and 1600 Amperes (A) are commonly used in sub-sea power cables which would consequently produce a surface EMF of up to 3.20 millitesla (mT) (1600A) in a perfect wire (Bochert and Zettler, 2006). Previous studies have highlighted that there is insufficient data to allow informed assessment of EMF emissions around MRED cable deployments (CMACS, 2003). Values used in previous studies are highly variable, for example; 3.7mT (Bochert and Zettler, 2004), 4.2mT (Formicki *et al.,* 2004), 36mT (DC) and 165mT (AC) (Cada *et al*., 2011), 3mT (Woodruff *et al.,* 2012), 2.8mT and 40mT (Scott *et al.,* 2018a).

The European lobster, *Homarus gammarus*, and the edible crab, *Cancer pagurus*, are commercially important decapod crustaceans found throughout Western Europe (Holthius, 1991; Scott *et al.,* 2018b) from Scandinavia to North Africa (Eastern Inshore Fisheries and Conservation Authority, 2017). *H. gammarus* and *C. pagurus* are of high commercial value and fished throughout western Europe (see Chapter 1.). Both species have been shown to be present around MREDs and have shown attraction to EMF of the strengths predicted around subsea power cables (see Chapter 4. and 5.). Both species have also been shown to exhibit physiological and behavioural changes during exposure to increased EMF (see Chapter 4. and 5.).

The study of reproduction in crustaceans can be problematic due to the unpredictable occurrence of receptive females, infrequent mating and female crustaceans' ability to produce several clutches from a single mating, making paternity hard to assess (Christy, 1987). In crustaceans, after spermiogenesis, sperm is encapsulated in acellular secretions, thus creating a spermatophore (Kooda-Cisco and Talbot, 1983). The spermatophores are then passed to the females during copulation via the male gonopod and stored until spawning and fertilization (Fasten, 1917; Matthews, 1954a,b).

Mating in *H. gammarus* occurs in late summer early autumn, after moulting has taken place, resulting in the females producing an egg mass of approximately 14,000 eggs (Lizarraga-Cubedo *et al.,* 2003). The moulting cycle in lobsters typically takes place over the summer months with a single moult per year, becoming less frequent in older animals (Eastern Inshore Fisheries and Conservation Authority, 2017). Intermoult mating has been shown to occur in the American lobster, *Homarus americanus*, where females are looking to replenish their sperm stores, or were unsuccessful in mating post moult (Waddy *et al.,* 1995; Atema and Steinbach, 2007; Skog, 2008). Despite a lack of evidence, this may be the case with *H. gammarus* given the similar life cycle. *H. gammarus* females carry eggs on their pleopods for a period of 9-11 months, sometimes longer (Branford, 1978).

As with *H. gammarus*, *C. pagurus* moult once per year between summer and early winter, with moult frequency decreasing in older animals. Mating occurs post moult with the male guarding the soft-bodied female for several days either side of the event for protection (Edwards, 1979). Male crabs are polygamous and often attend several females during the breeding season (Edwards, 1979). Mature female *C. pagurus* have been shown

to migrate considerable distances offshore to lay their eggs (Williamson, 1900; Ungfors *et al.,* 2007; Hunter *et al.,* 2013). The females move to deeper waters to lay and incubate their eggs in areas where the substrate is fine enough to dig a pit (Nichols *et al.,* 1982). Once berried, females will spend the next 6-9 months half buried in these pits with very little movement and significantly lower feeding rates (Williamson, 1900; Edwards, 1979; Howard, 1982; Naylor *et al.,* 1997). *C. pagurus* fecundity has been linked to carapace width, with larger crabs producing more eggs (0.5 – 3 million eggs) (Williamson, 1900; Edwards, 1967; Ungfors, 2007).

In *C. pagurus*, after around 8 months when the eggs have reached full development (progressed through the 6 stages identified by Chung *et al.* (2004) in *Carcinus maeanas*, including yolk, embryogenesis, eye enlargement, heartbeat detected, developing eye, and full development) they will begin to hatch as protozoea larvae before progressing through 5 zoeal stages resulting in the final larval stage known as megalopa. Once megalopa the larvae will begin to settle from the water column to the benthos within 24 hours (Edwards, 1979). Information on *H. gammarus* egg development is lacking in the literature, however comprehensive studies have been conducted on the closely related *H. americanus* (Helluy and Beltz, 1991). In this study lobster egg development was described in detail from freshly laid eggs to 100% development which took a period of 159 days in total. Larval hatching typically occurs during the night for lobsters, lasting for successive nights for periods of 2 – 6 weeks (Ennis, 1973). Larvae then have a pelagic phase (zoea I – IV) of around 6-8 weeks pre-settlement with potential to travel extensive distances along ocean currents (Huserbraten *et al.,* 2013).

Previous studies have highlighted that both species, in addition to other marine organisms, are found to inhabit areas around MREDs, particularly scour protection zones which can act as artificial reefs (Langhamer and Wilhelmsson, 2009; Hunter and Sayer, 2009; Hiscock *et al.,* 2010). Both *H. gammarus* and *C. pagurus* have been found to be attracted to EMF at strengths predicted to occur around sub-sea power cables (Scott *et al.,* 2018a, Chapter 5.). Given the reduction in movement of this species during the incubation period, combined with this attraction, suggests that a large portion of egg development could take place under increased EMF exposure.

The effects of EMF on egg development and larval locomotory ability of both *H. gammarus* and *C. pagurus* are currently unknown. The aims of this chapter are to assess

the effects of EMFs, at strengths predicted around offshore windfarms, on egg development and subsequent larval morphology and locomotor ability of these two highly important species.

6.2 Materials and Methods

A total of 20 ovigerous female lobsters, ≥MLS (87mm carapace length) were caught within the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK) by local fishers and immediately transported to the research facility. Lobsters were housed in individual 500 L flow-through tanks until use. Tanks contained small pebbles as substrate, received a constant supply of raw unfiltered, ambient temperature sea water and natural light conditions. Lobsters were fed live blue mussel twice a week during holding and experimentation with uneaten food being removed after 24 h. Prior to experimentation all lobsters were subjected to a conditions index modified from Scott *et al.* (2018a), ensuring only those categorized as grade 1 or 2 were used for experimentation, with surplus lobsters being released. Lobsters utilised for experimentation were of a similar weight (584.71±108.9 g) and carapace length (92.33±4.3 mm). The egg mass of the chosen females was deemed similar by way of a visual inspection.

A total of 25 adult female *C. pagurus* with sperm plugs were collected by local fishermen caught in the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK) in Autumn 2017. Female crabs were housed in several large holding tanks (30,000 – 100,000 L) with natural substrates, a constant supply of raw unfiltered, ambient temperature sea water and natural light conditions. Crabs were fed on a natural diet of blue mussel, squid, and fish during holding and experimentation with uneaten food being removed after 24 h. Crabs were visually assessed weekly for signs of egg laying. Once a female had finished laying eggs, their condition index from Scott *et al.* (2018a) was determined and only crabs with grade 1 or 2 were used for this study. Female crab's carapace width (all above MLS (≥140 mm),157.8±8.8 mm) and wet weight (704.92±211.5 g) were measured prior to the start of experimentation. Experimentation was conducted between January 2018 – July 2019.

Experimental Setup

A total of 6 berried female lobsters and 6 crabs per treatment (EMF and control) were placed in 30 L glass tanks containing a black ABS shelter, individual air stones and a sterilised pebble substrate for lobster and fine sand for crabs. All tanks received ambient temperature flow-through seawater (flow rate 0.5 l min^{-1}). Temperature (7.6-14.6±0.1 °C)), salinity (34±0.20 ppt) and dissolved oxygen (>90%), were monitored throughout the experiment and kept constant (YSI ProDSS multiprobe and HOBO UA-002-08 8k temp/light pendant). All animals received natural light conditions via a transparent roof on the aquarium. Each glass tank was situated inside a 50 L black ABS box which acted as a water bath to ensure no significant temperature changes occurred throughout the experiment and to reduce visual stimuli.

In EMF treatment 6 berried female crabs and 6 lobsters were exposed to a constant uniform static DC EMF (2.8mT), created by Helmholtz coils for the duration of egg incubation. The EMF strength was chosen as it is the value predicted to be present around subsea power cables (Bochert and Zettler, 2006; Scott *et al.,* 2018a). The EMF within the coil was mapped using a recently calibrated AlphaLab Inc, Gaussmeter Model GM-2 and was found to be uniform between experimental tanks placed in the test arena. Helmholtz coils were powered using a variable DC power supply (Elektro-Automatik EA-PSI 8360-15). In control groups 6 berried female crabs and 6 lobsters were placed in an identical experimental arena described above, but the Helmholtz coils remained unpowered throughout the egg incubation period.

Egg development

Crabs were placed into the experimental tanks once they had finished laying eggs which varied between individuals by up to 4 weeks. All crabs used in experiments began trials in January 2018, with eggs in identical developmental stages (stage 1), using the developmental stages previously described by Chung *et al.* (2004). In order to standardise the egg development between berried lobsters, experimentation began in April 2018, when all lobster eggs were at 50% development, based on the developmental stages described by Helluy and Beltz (1991).

Lobster eggs were sampled periodically (first two samples monthly, and weekly thereafter) throughout embryonic development. Female lobsters were removed from their

tanks and a small portion of eggs were removed from the pleopods using a sterile spatula. Total egg sampling time was under 30 seconds to avoid inducing stress in the females. The wet weights of 20 eggs were measured and the average single egg weight was calculated. The eggs were analysed for each female under a microscope (Leica MZ125, camera Brunel Eyecam Plus) to determine the developmental percent-stages based on eye index by Helluy and Beltz (1991), and to screen for deformities. Eggs were photographed and images were post-processed in ImageJ (Version 1.52A) (Schneider *et al.*, 2012). Egg volume was calculated based on the formula of oblate spheroids: $V = 1/6(\pi W^2 L)$, where V is the egg volume (mm^3), W is the minimum diameter of the egg (mm) and L is the maximum diameter of the egg (mm) (Lei *et al*., 2014). The same procedure described above for *H. gammarus* was utilised for egg sampling in *C. pagurus*. Crab eggs were sampled throughout embryonic development (first three sampling monthly thereafter every 10 days). Sampled eggs were photographed with compound microscope (Leica DM750, camera DMC 5400), staged based on Chung *et al.* (2004), measured and screened for deformities. Egg volume was calculated using the same formula as described above.

Larval assessment

The dates of hatching were recorded for each female lobster and crab. After hatching started each tank was put on a temperature-controlled recirculating system, with daily water change to avoid losing larvae through outflows. As hatching occurs mainly at night, all freshly hatched stage I, per Hadley (1908), lobster larvae and stage I zoea crab, per Ingle (1981), larvae were collected at 09:00 am every day and counted throughout the hatching period (Ennis, 1973). Due to the large number of *C. pagurus* larvae hatching daily, larvae were carefully collected in a filter to avoid damage and counted from 5 subsamples to estimate density and subsequently the total number of larvae released. The larval release duration was recorded for each crab and lobster. A total number of 100 freshly hatched larvae from each female were individually photographed under a dissection microscope (Leica MZ125, camera DMC 5400). The images were then analysed with ImageJ to obtain measurements of larval parameters. Total length, carapace height, carapace length, and maximum eye diameter (Figure 6.1.) were measured on lobster larvae, while dorsal and rostral spine length, carapace height and length, total length and maximum eye diameter were measured on crab larvae (Figure

6.1.). A total of 2651 EMF exposed and 3573 control stage I lobster larvae were screened for deformities such as curled or bent rostrum, swollen carapace, twisted abdomen, damage of tail fan, twisted pereopods, misshapen claws and chromatic aberrations in the eye (Agnalt *et al*., 2013). In total, 2703 freshly hatched, EMF exposed larvae and 3701 control crab larvae were screened for the same deformities as above and additionally for rostral, dorsal and lateral spine deformation.

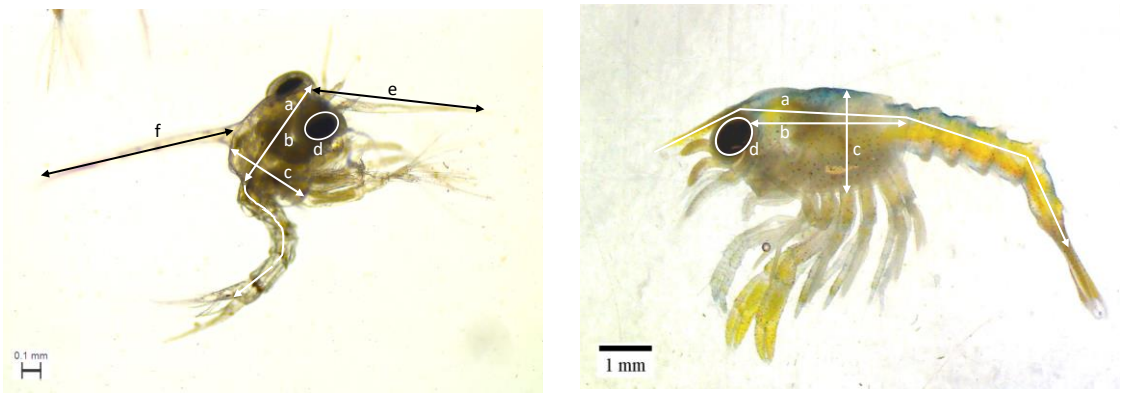


Figure 6.1. Zoea I *C. pagurus* larvae (left) and stage I *H. gammarus* larvae length measurements: a) total length (TL), b) carapace length (CL), c) carapace height (CH), d) maximum eye diameter, e) rostral spine length (RSL) , f) dorsal spine length (DSL). Photo: St Abbs Marine Station.

Vertical swimming speed

Freshy hatched lobster larvae and crab larvae show positive phototaxis (Botero and Atema, 1982; Webley and Connolly, 2007). Larvae from each female were collected and were subjected to experiments to determine vertical swimming speed based on the method of Schmalenbach and Buchholz (2009). Before sunrise larvae were collected and transferred to a separate acclimating holding tank with ambient salinity, temperature, aeration and kept in a temperature controlled dark room prior to the trial. The experimental chamber consisted of 75 ml (lobster larvae) and 15 ml (crab larvae) black glass vertical cylinders with a clear window at the base. The cylinders were filled with UV sterilised, filtered (0.22 µm) seawater at the same temperature as the holding tank. The bottom of the cylinder was lit at the window using a handheld torch and the time taken for the individual larvae to sink down (sinking rate) was measured. This light was

then switched off and the light at the top of the cylinder was switched on. The time was measured for the individual larvae to reach the top of the cylinder without stopping, turning or sinking back. The vertical swimming speed was calculated by the difference between the measured speed and sinking rate in cm s^{-1} and mm s^{-1}. Larvae were only used once, regardless of the trial success. Trials where the larvae failed to swim up after 5 mins were classed as a failed trial.

Statistical analysis

Data is presented as the mean and standard error (SEM) of replicates. The statistical analysis was performed with the computer programs SPSS (IBM SPSS Statistics v.23 SPSS Corp. Chicago, USA) and STATISTICA version 7.1 (StatSoft Inc., 2005). The data was tested for parametric assumption by Kolmogorov-Smirnov test and Levene's test. When data met these assumptions, they were subjected to one-way or two-way analysis of variance (ANOVA), multivariate analysis of variance (MANOVA) followed by post-hoc analysis of Tukey's test. If data did not meet parametric assumptions, non-parametrical analysis of Mann-Whitney U-test was performed with Bonferroni correction where appropriate. To test difference of prevalence of larval deformities and mortality between treatments, pairwise comparisons by Chi-squared tests with Yates correction were performed. All statistics were tested at a probability of 0.05. Statistical differences of data sets are indicated on the presented graphs by single ($p<0.05$), double ($p<0.01$) or triple ($p<0.001$) asterisks.

6.3 Results

Egg development

Homarus gammarus

The total time of full embryonic development, from 50% development to hatching did not differ significantly between EMF and control lobsters. The mean time taken from 50% to date of first hatch under control conditions was 82.8±9.8 days (N=5), whilst during EMF exposure the time taken to first hatch was 69.7±7.3 days (N=6) (Table 6.1.). During experimentation one control lobster's eggs reached 80% development then

stopped advancing and were subsequently dropped by the female, therefore this data was omitted from further statistical analysis.

Egg volume increased by approx. 2 µl from experimental start (50%) to hatching in both treatments. At 60% development there was a significant increase (6.37%, $t(514)=-5.87$, $p<0.05$, t-test) in the EMF exposed egg volume when compared to the control. At 70 % there were no differences in egg volume between the two treatments. At 80% and 90% development there were significant decreases in EMF exposed egg volumes (13.5% and 15.5% respectively, $t(295)=4.32$ $p<0.05$, $t(39)=4.90$, $p<0.01$, t-test). At 100% development, control and EMF exposed egg volumes showed no significant differences.

Table 6.1. Embryonic development time (days) for each lobster and crab under control and EMF exposed conditions. *H. gammarus* N=11, *C. pagurus* N=11.

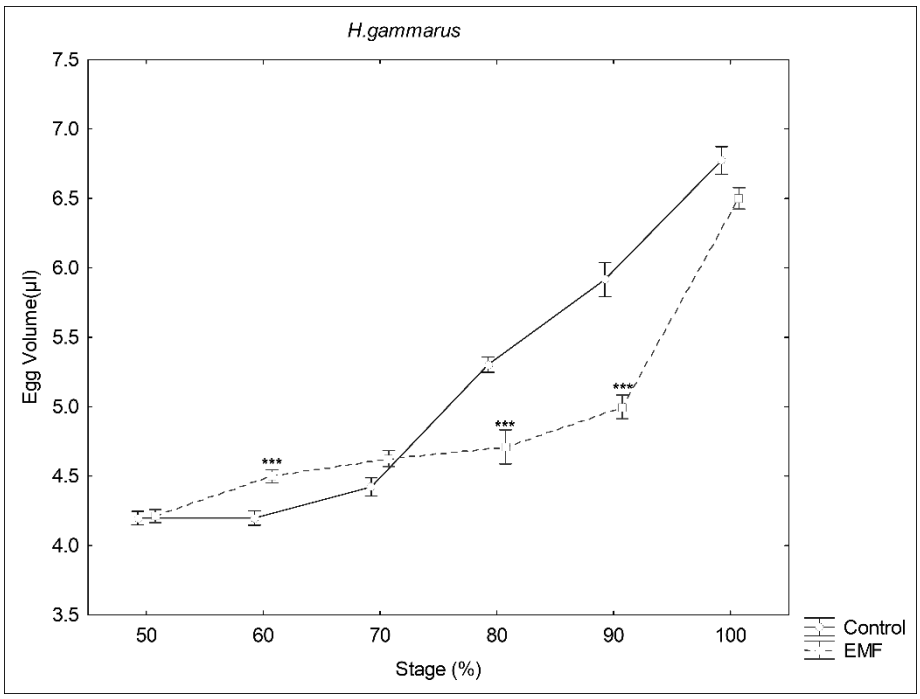
	H. gammarus		*C. pagurus*	
Females	Control	EMF	Control	EMF
1	63	80	N/A	113
2	N/A	61	112	113
3	55	57	106	102
4	96	68	133	133
5	99	100	106	145
6	101	52	107	137

Exposure to EMF did not have a significant effect on egg weight between 50% - 100% development. Egg weight increased from 4.05±0.1 mg at 60% to 5.71±0.25 mg at 100% with no significant deviation between treatments. There were no embryonic deformations recorded throughout the egg development trials.

Cancer pagurus

During experimentation one control crab's eggs reached stage 2 then stopped advancing, followed by the death of the female, therefore data obtained from this crab was omitted from statistical analysis. The total time of embryonic development, from stage 1 to

hatching did not differ significantly between EMF exposed (124±7 days) and control crabs (113±5 days) (Table 6.1.). Egg volume significantly increased throughout embryonic development by an average of 0.025 mm^3 from stage 1 to stage 6 in both treatments ($F(5,1915)=646.02$, $p<0.001$, one-way ANOVA). Between stage 2 and 4 egg volumes were significantly smaller in EMF exposed crabs compared to control, at stage 5 there were no differences between the two treatments, and stage 6 EMF exposed crab's egg volume was significantly larger compared to control ($F(1,438)=19.39$, $p<0.001$, 2-way ANOVA, Tukey's posthoc test).



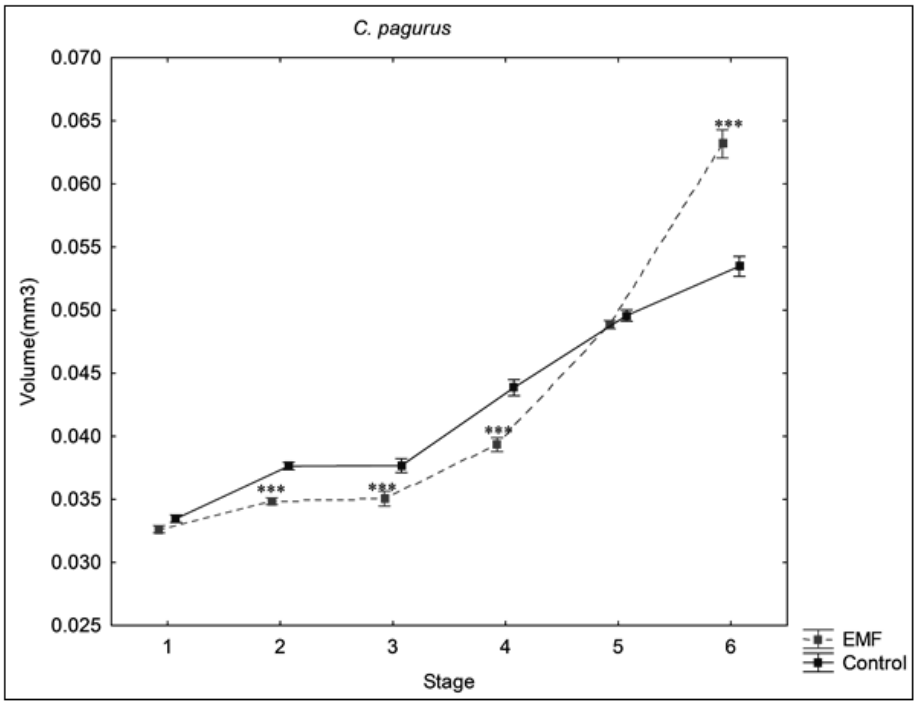


Fig. 6.2. Egg volume (mm^3) throughout embryonic development of *H. gammarus* (50% to 100% development scale) and *C. pagurus* (stage 1 to stage 6) in EMF exposed (red) and control groups (blue). Markers indicate mean values, vertical bars denote +/- standard errors. Significance levels between two treatment at each stage indicated by asterisk, * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. *C. pagurus* N=1920, *H. gammarus* N=2560.

Hatching

Homarus gammarus

The total number of hatched larvae ranged from 136 – 3812 under control conditions and 76 – 5062 under EMF conditions. Under control conditions a total of 3 (60%) lobsters successfully released over 2000 larvae whilst only 2 (33%) EMF exposed lobsters had a success rate of over 2000. The mean total number of days taken from first hatch to final release of larvae was slightly higher for EMF exposed lobsters (18.2 ± 1.1 days) compared to under control conditions (14.4 ± 1.4 days), although this difference was not statistically significant.

Cancer pagurus

The total larvae hatched ranged from 154,368 to 1,672,020 larvae under control conditions and between 94,985 and 673,670 larvae in EMF exposed crabs. The lower number of hatched larvae in EMF exposed crabs was not statistically significant. Under control conditions 50% of crabs successfully released over 500,000 larvae whilst only 20% of EMF exposed crabs had a similar success rate.

There were no significant differences between the mean total number of days taken from first hatch to final release of larvae in EMF exposed crabs (8.8±1.4 days), compared to crabs kept in control conditions (8.3±1.2 days).

Larval measurements

Homarus gammarus

Deformities were significantly higher in EMF exposed larvae compared to control ($P<0.001$, Chi-square with Yates correction). Of the 2651 EMF exposed larvae assessed, 3% showed carapace, abdominal, rostral and tail deformations, compared to 1% of the 3573 larvae assessed under control conditions (Figures 6.3., 6.4.). Mortality rate was significantly lower in EMF exposed larvae (0.01%), compared to the control group (0.24%) ($p<0.01$, Chi-square with Yates correction).

Larvae exposed to EMF throughout their embryonic development had a significantly smaller total length (TL), carapace height (CH) and maximum eye diameter (ED) and a significantly longer carapace length (CL) ($F(1,6222)=3.9, 100.9, 118.98, 63.75$, $p<0.001$, MANOVA) (Figures 6.5., 6.6., 6.7., 6.8.).

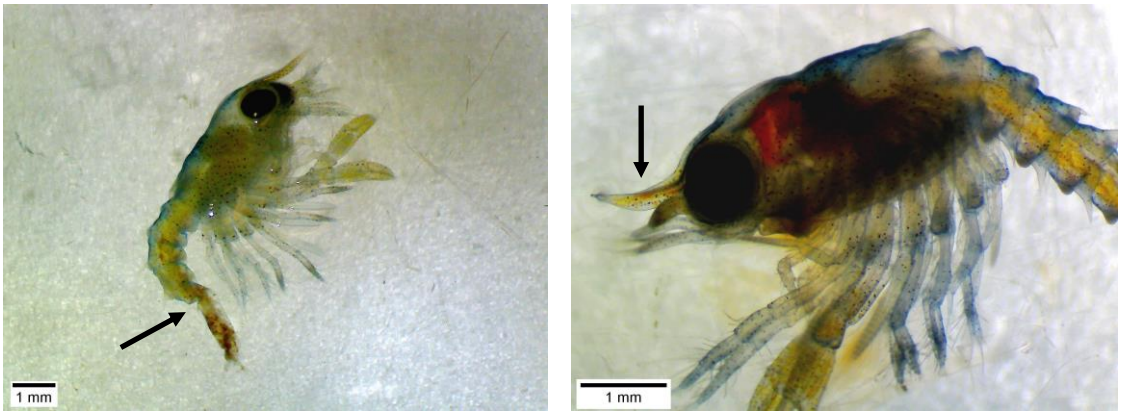


Figure 6.3. Deformities observed in freshly hatched Stage I *Homarus gammarus* larvae, exposed to 2.8 mT EMF throughout their embryonic development. Left image shows a larva with tail deformation, indicated by black arrow. The image on the right shows a larva with a bent rostrum. Photo: St Abbs Marine Station.

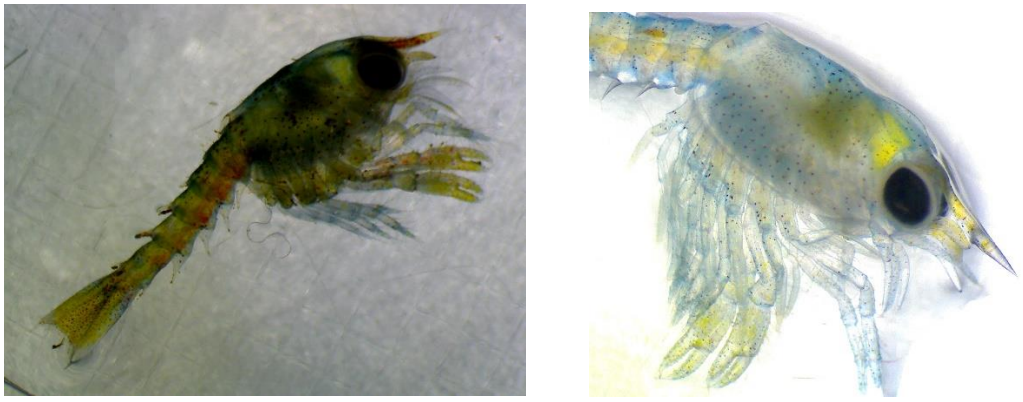


Figure 6.4. For comparison, the image on the left showing a fully formed larval tail and rostrum on the right image. Photo: St Abbs Marine Station.

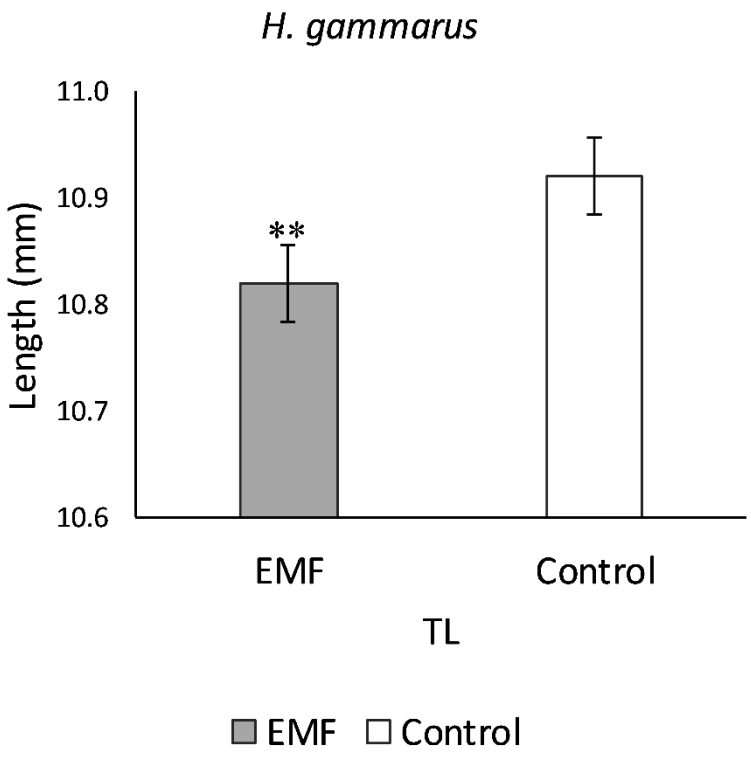


Figure 6.5. Total length of EMF exposed *H. gammarus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2651$, $N_{CONTROL} = 3573$.

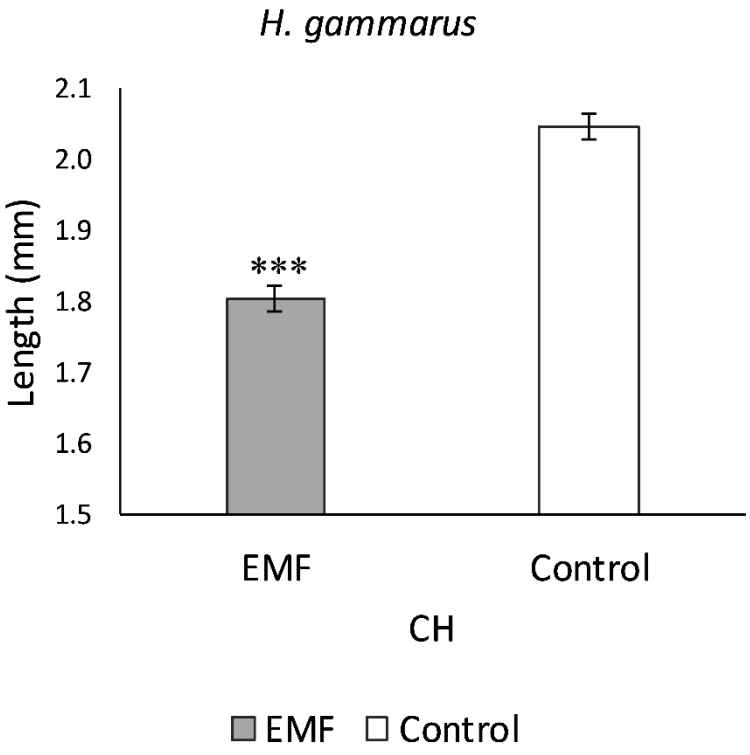


Figure 6.6. Carapace height of EMF exposed *H. gammarus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2651$, $N_{CONTROL} = 3573$.

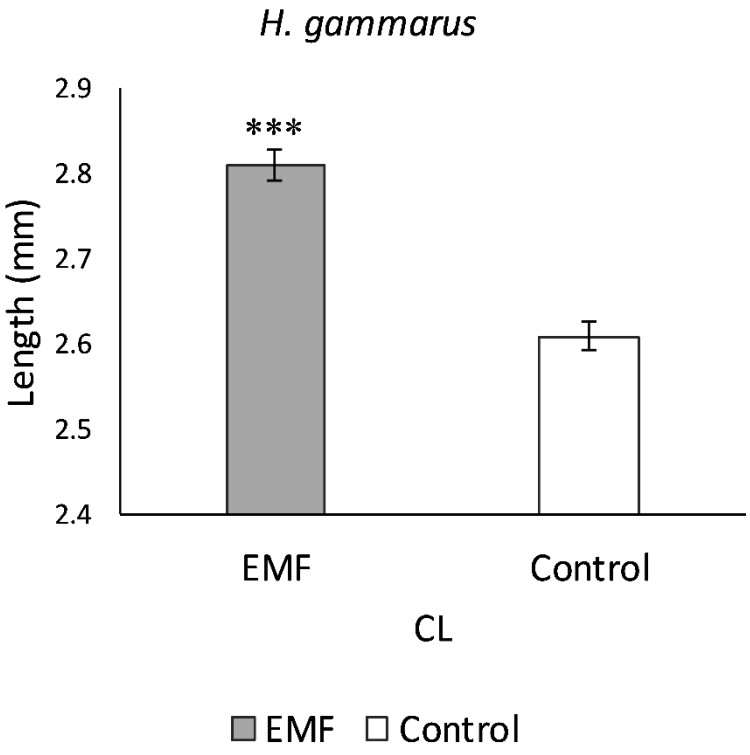


Figure 6.7. Carapace length of EMF exposed *H. gammarus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2651$, $N_{CONTROL} = 3573$.

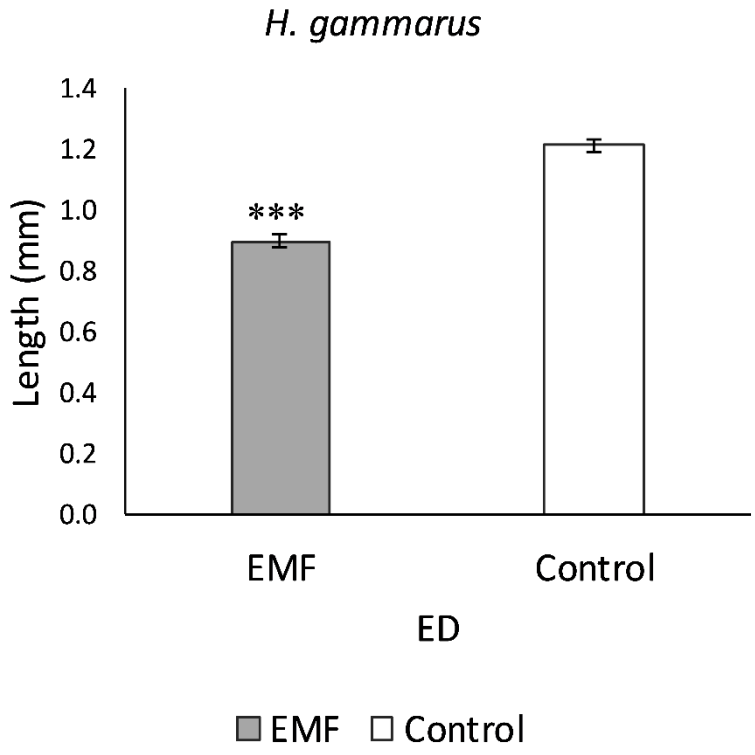


Figure 6.8. Eye diameter of EMF exposed *H. gammarus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2651$, $N_{CONTROL} = 3573$.

Cancer pagurus

Larval deformities in both conditions occurred in low quantities (<1%) and did not differ significantly. EMF exposure did not affect the mortality rate of freshly hatched larvae, with 11.1% in EMF exposed crabs and 14.3% in control conditions. Larvae exposed to EMF throughout their embryonic development were significantly smaller in all the measured parameters (RSL ($F(1,6402)=19.38$, DSL $F(1,6402)=52.45$, CH $F(1,6402)=9.9$, CL $F(1,6402)=32.94$, TL $F(1,6402)=42.05$, $p<0.001$, ED $F(1,6402)=42.05$, $p<0.05$, MANOVA) (Figures 6.9., 6.10., 6.11., 6.12.).

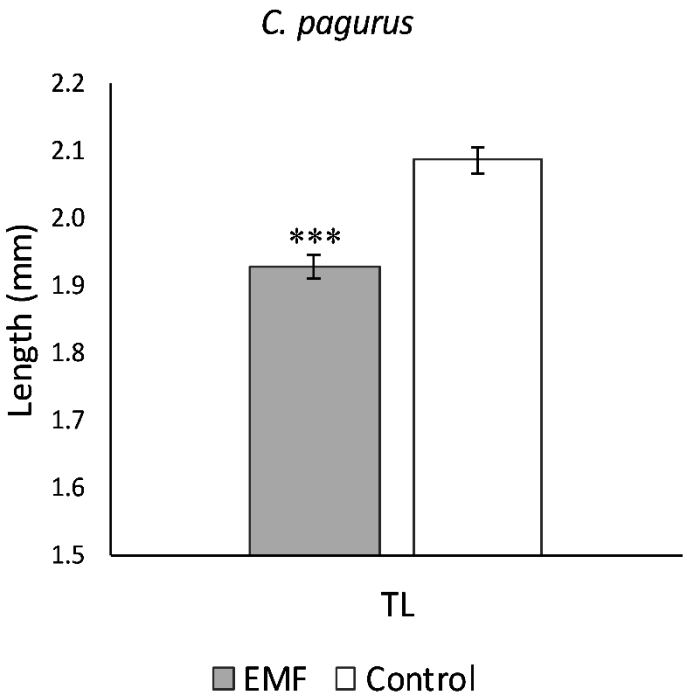


Figure 6.9. Total length of EMF exposed *C. pagurus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2703$, $N_{CONTROL} = 3701$.

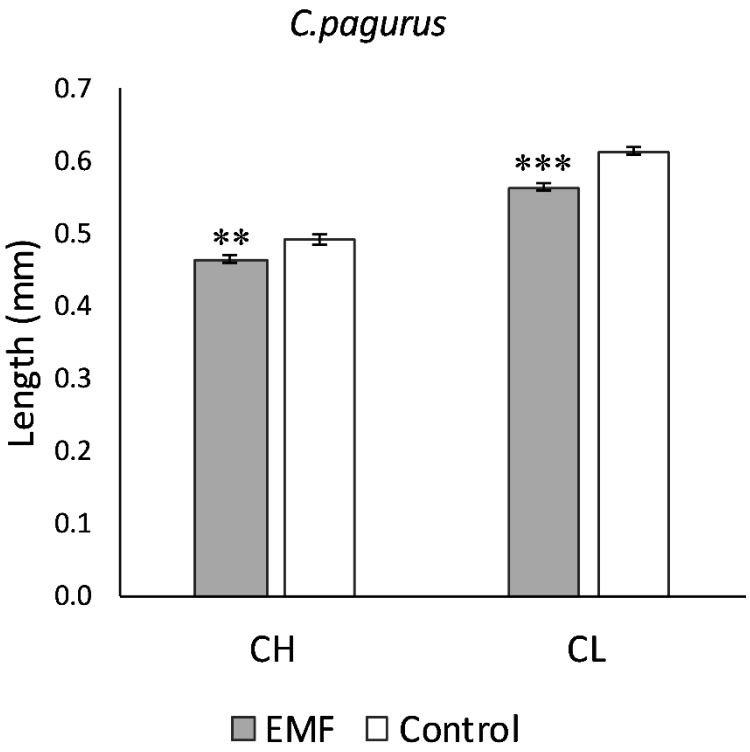


Figure 6.10. Carapace height and carapace length of EMF exposed *C. pagurus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2703$, $N_{CONTROL} = 3701$.

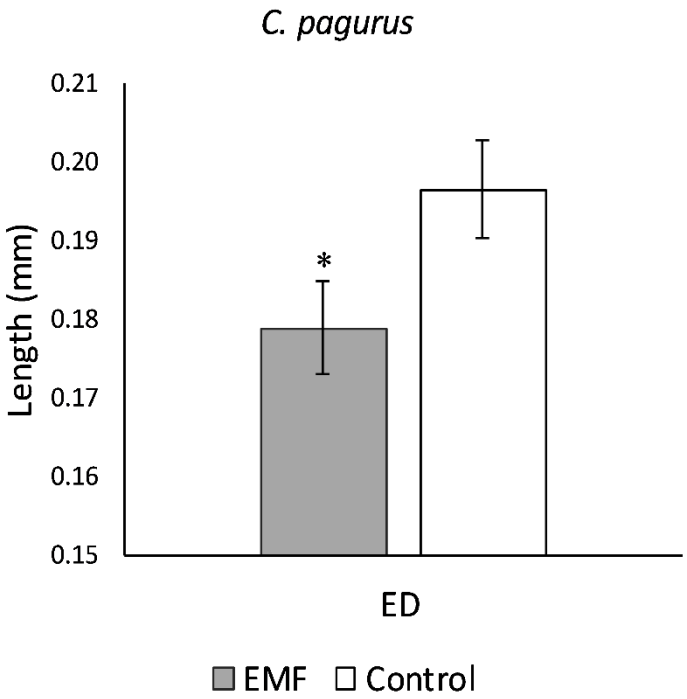


Figure 6.11. Eye diameter of EMF exposed *C. pagurus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2703$, $N_{CONTROL} = 3701$.

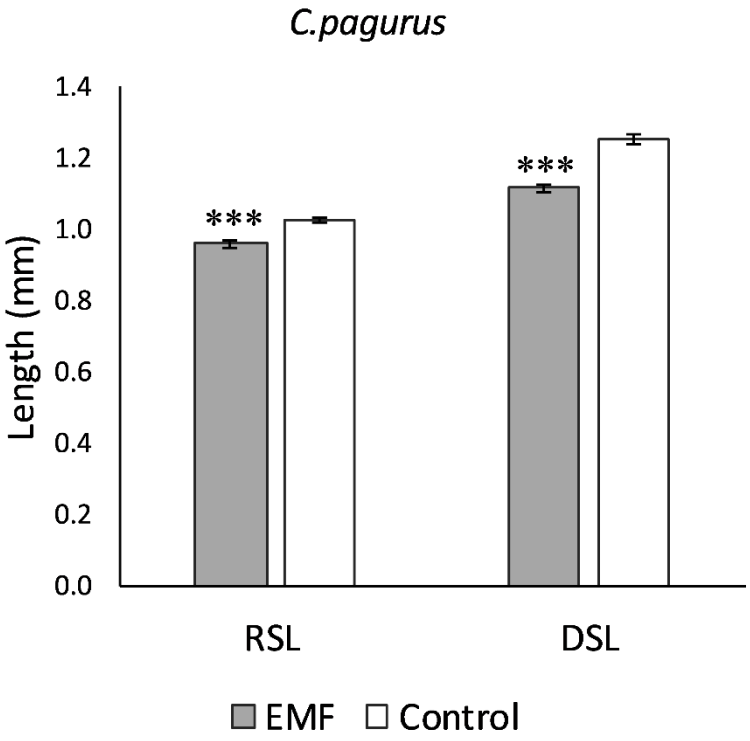


Figure 6.12. Rostral spine and dorsal spine length of EMF exposed *C. pagurus* larvae compared to control larvae. Bar graphs indicate mean values of each measured parameter, vertical bars denote +/- standard errors. * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. $N_{EMF} = 2703$, $N_{CONTROL} = 3701$.

Vertical swimming speed

Homarus gammarus

A total of 151 EMF exposed larvae (and 106 control) were subjected to locomotory tests. There were significantly more failed trials (larvae failed to reach the top of the test chamber after 5mins) in the EMF exposed larvae (11%) than those reared under control conditions (4%) ($p<0.05$, Chi-square with Yates correction). Of the successful trials, which ranged from 0.23 to 18.9 cm s^{-1} in EMF larvae and 0.13 to 19.6 cm s^{-1} in control larvae there were no significant differences found. The mean vertical swimming speeds did not differ significantly between EMF exposed larvae (2.22±0.3 cm s^{-1}) and the control group (2.39±0.4 cm s^{-1}).

Cancer pagurus

A total number of 60 EMF exposed, and 129 control larvae were used in locomotory tests. There was no significant difference between the percentage of failed trials between EMF (55%) and control groups (64%). EMF exposure during embryonic development did not significantly affect the swimming speed of freshly hatched larvae with an average of 3.3±0.5 mm s^{-1} in the EMF exposed group and 1.6±0.4mm s^{-1} in the control.

6.4 Discussion

The results obtained throughout this study have shown that exposure to increased EMF, at strengths predicted around subsea power cables, has a significant impact on egg volume, and larval morphology, resulting in smaller larvae across most measured parameters in both *H. gammarus* and *C. pagurus*. A higher number of deformities and a decrease in larval locomotory abilities in *H. gammarus* larvae was also observed.

The average number of days from 50% development to hatching in lobsters (82.8±9.8 days in EMF, 69.7±7.3 days in control) and stage 1 to hatching in crabs (124±7 days EMF, 113±5 days) control coincides well with those found in literature. Wear (1974) found that *C. pagurus* embryonic development was approximately 80 days from end of diapause, which can be up to 60 days in duration. In a study by Charmantier and Mounet-Guillaume (1992) the average embryonic development time in *H. gammarus* from 50% development ranged between 60 to 80 days. Hatching in *H. gammarus* ranges from 7 to 10 days according to Charmantier and Mounet-Guillaume (1992). In this study the average hatching duration of European lobster was slightly longer (18.2 ± 1.1 days in EMF, 14.4 ± 1.4 days in control). Hatching in *C. pagurus* according to Edwards (1979) is in agreement with our findings (8.8±1.4 days EMF, 8.3±1.2 days control). Mean hatching times were slightly higher in EMF exposed groups in both species, however due to high individual variation between females these differences were not significantly different.

Zimmerman *et al.* (1990) reported that exposure of fertilized sea urchin eggs to a 60Hz (AC), 0.1mT EMF delayed development whilst Cameron *et al.* (1985) also found developmental delays in fish embryos. Differences in our findings highlight the different sensitivity of species to EMF exposure. High variability of embryonic development time

and hatching in individual females makes it difficult to standardise and may also have accounted for differences within the results obtained within this study. Further studies are required utilising a higher number of replicates to fully understand the effects of static EMF on these species' egg development timescales.

Long-term exposure to 2.8mT EMF during egg incubation period, showed stage specific negative effects on egg volumes in both *H. gammarus* and *C. pagurus*. The eggs of marine invertebrates increase their volume significantly during embryonic development by a slow but steady osmotic uptake of water (Davis, 1968). *C. pagurus* and *H. gammarus* egg volume increased by approximately 50% during embryonic development in this study which agrees with results found by Wear (1974) and Helluy and Beltz (1991). It has been reported that the rate of water uptake begins around the time the embryonic heartbeat is first observable, as the volume of the eggs increases more rapidly from this time (Wear, 1974). Studies have shown that the cell membrane is one of the main targets of static EMF in cells, increasing membrane permeability, changing membrane dynamics and embedded proteins, such as ion channels, responsible for maintaining cell osmosis (Petrov and Martinac, 2007). Disrupted egg membrane permeability could account for changes found in EMF exposed egg volumes in both species.

EMF exposure showed significant effects on all measured larval parameters. Stage I lobster larvae from EMF exposed females showed smaller total length, carapace height and eye diameter, but a longer carapace length. EMF exposed *C. pagurus* larvae had a decreased rostral and dorsal spine length, carapace length, total length and maximum eye diameter. Larval size can be negatively affected by several environmental factors such as salinity, temperature, and photoperiod, (Fonds, 1979; Gardner and Maguire, 1998). Keppel *et al.* (2012) found decreased carapace length with increased pCO_2 levels in the closely related *H. americanus*, and the moulting cycle was prolonged by about 2 days in larvae. Zahedi *et al.* (2014) found that exposure to EMF resulted in decreased weight of mice pups, whilst other studies found increased size of chickens (Rooze *et al.*, 1985) or no difference in EMF exposed American kestrel, *Falco sparverius,* (Kernie *et al.*, 2000).

Deformities were observed in hatched larvae of both species, however only *H. gammarus* showed significantly increased occurrences in the EMF exposed treatment, with 3% of hatched larvae developing deformities. Mortality rate of zoea I crab larvae was not affected by EMF exposure, whilst EMF exposed stage I lobster larvae showed a

significantly lower mortality rate when compared to control group. Deformities in marine invertebrate larvae were reported to affect adult individual mortality and egg production (Agnalt *et al.*, 2013). In the present study, the most frequent deformities in the stage I lobster larvae in EMF treatment were found in the carapace, tail fan and/or in the rostrum. Similar deformities due to elevated pCO_2 levels have been documented on the embryonic and larval stages in other marine invertebrate species, (Kurihara, 2008; Kawaguchi *et al.*, 2010; Gibson *et al*., 2011). Deformities generally affect carapace, walking legs, claws, abdomen, tail fan and even antenna with those affected usually developing multiple deformities (Agnalt *et al.*, 2013). Agnalt *et al.* (2013) found that lobster larvae exhibiting damage to the tail fan could not be repaired through subsequent moulting. Adverse effects of EMF on ontogenesis in other species have been also described. For example, Giorgi *et al.* (1992) reported an increased mutation rate in fruit flies, *Drosophila*, exposed to a magnetic field of 2.5mT and Ueno *et al.* (1984) found African clawed toad embryos exposed to 1T EMF resulted in reduced pigmentation and axial anomalies. The high ratio of lobster larvae with deformed exoskeletons strongly indicates a negative effect of exposure to 2.8 mT EMF on *H. gammarus*. Some of the deformities may affect respiration (carapace), and locomotor ability (tail-fan damage).

Positive phototactic behaviour was negatively affected in 2.8mT EMF exposed stage I lobster larvae, with 7% more failed trials. Neither *C. pagurus* zoea I larvae vertical swimming test success, nor vertical swimming speed of freshly hatched lobster and crab larvae were affected by EMF exposure during embryonic development.

Our findings of an average vertical swimming speed of 2.39±0.4 cm s^{-1} in *H. gammarus* and 1.6±0.4mm s^{-1} in *C. pagurus* correspond with values in literature. Schmalenbach and Buchholz (2009) measured a maximum swimming speed of 2.9±0.5 cm s^{-1} in *H. gammarus* larvae, Foxon (1934) found that squat lobster, *Galathea dispersa*, maximum swimming speeds was 1.79 cm s^{-1} and Guo *et al.* (2012) determined swimming speeds of 0.15 ± 0.008 mm s^{-1} in *Brachionus Calyciflorus*.

Newly released stage I larvae, of both species, show a strong positive phototaxis resulting in a preference for the uppermost water layers with high food abundance and surface currents, that carry the larvae to suitable areas of settlement (Scarratt, 1964; Nichols, 1982). Larval swimming ability plays an important role in maintaining position within currents (Ennis, 1986). When larvae stop swimming by beating of the exopodites, they

sink towards the bottom, where currents are reduced (Hadley, 1908). This positive phototactic reaction was observed in the early phase of shore crabs, *Carcinus maenas*, and the Harris mud crab, *Rhithropanopeus harrisii*, larvae (Sulkin, 1984). Failure of this positive phototaxis response combined with the reduced swimming ability in EMF exposed lobsters may negatively impact larval recruitment processes. Acute exposure to EMF has been observed to disrupt orientation and locomotory behaviour in several studies, e.g. in zebrafish, *Danio rerio*, (Ward *et al.*, 2014), in fruit fly, *Drosophila melanogaster*, (Fedel *et al.*, 2014), *Talorchestia martensii* (Ugolini, 2006) and in *H. americanus* (Hutchison *et al.*, 2018). This study concludes that long-term exposure to EMF during embryonic development can also affect stage I larval phototactic behaviour. Differences found in the test failure ratio of EMF treated *H. gammarus* could also be accounted for by the elevated prevalence of lobster larval deformities.

Different marine species have different EMF sensitivity ranges, therefore accurate measurements of strengths around submarine power cables are essential in order to accurately assess the effect and impacts of MREDs on marine benthic fauna. Cumulative effects of MRED related stressors on marine invertebrates, such as current alterations around wind turbines, increased sea temperature around cables and substations, and operational underwater sound must be investigated in the future on all life stages of inhabiting organisms. The effect of temperature is particularly important, as ovigerous female lobsters move offshore to reduce thermal variability during nursing periods (Campbell and Stasko, 1986). Ovigerous *C. pagurus* will likely be attracted to EMF emitted from subsea power cables (Scott *et al.*, 2018a). Further work must be undertaken to build upon the results found in this study in order to accurately determine the future implications of the effects found to occur during EMF exposure on these species' larval development. Different sensitivities to EMF may explain the different effects found within the two species. Further studies on gene expression, larval physiology, growth, survival and feeding rate is needed to fully understand the impact of offshore renewable energy devices on the reproductive cycle of *H. gammarus* and *C. pagurus*.

Chapter 7. Behavioural and physiological responses in the Edible crab, *Cancer pagurus* (L.), to multiple strength electromagnetic field emissions from Marine Renewable Energy Devices (MREDs)

7.1 Introduction

Anthropogenically induced climate change through the burning of fossil fuels has a significant evidence base, which has led to many governments initiating programs for increased production of renewable or 'green' energy (Inger *et al.*, 2009). It is expected that with the current implications of climate change the number of marine renewable energy devices will increase, especially for locations that have the wind and wave resources (Inger *et al.*, 2009; Dannheim *et al.*, 2019). The increase in offshore renewables in Europe is expected to contribute to 10% of the continent's energy requirements by 2030 (Causon and Gill, 2018), with a current rise from 0.8 GigaWatts (GW) to 12.6 GW from 2006 to 2016 (Corbetta and Miloradovic, 2016; Causon and Gill, 2018). Marine Renewable Energy (MRE) promises to assist by providing clean, inexhaustible energy and aid in the reduction of GHG emissions (Boehlert and Gill, 2010). There are social and environmental concerns including habitat loss, collision risks, increased anthropogenic noise, exposure to increased electromagnetic fields (EMF) (Pelc and Fujita, 2002; Gill, 2005; Cada *et al.*, 2007; Boehlert *et al.*, 2008 Inger *et al.*, 2009; Scott *et al.*, 2018a). The continued assessment on the impacts of these structures is essential (Lindeboom *et al.*, 2015), as it has been highlighted that these energy devices are in planning to be partnered with sustainable seafood production sites (Holm *et al.*, 2017; Causon and Gill, 2018).

The number of subsea power cables connecting turbines, storage banks and export cables to shore will subsequently rise with the increase in deployments (Taormina *et al.*, 2018). These cables generate both an electric (E-field) and a magnetic (B-field) (Taormina *et al.*, 2018). Through industry standard insulation, E-fields can be successfully contained within the cable with no leakage, however there is no industry standard insulation that is able to prevent *B*-field leakage (Gill, 2005). The leaked *B*-field interacts with surrounding cable emissions, due to common cable configurations, leading to the creation of an induced electromagnetic field (iE-field) (see Chapter 1.) which is subsequently influenced by saltwater ions moving via underwater currents i.e. Lorentz force (De Luca, 2009).

There is a great variation in the strengths of the electromagnetic fields (EMF) arising from different structures, currents and cables (Normandeau *et al.,* 2011). Despite the large variations in strength, it is agreed that the highest strengths are likely to be found around the cables compared to turbine bases, particularly export cables (Thomsen *et al.,* 2015). EMF strengths predicted around subsea power cables as reported in the literature vary from 0.14mT – 8.02mT (Bochert and Zettler, 2006; Normadeau *et al.,* 2011; Cada *et al.,* 2011). Taormina *et al.* (2018) describes that a specific subsea cable running at 350 amperes (A) will generate a magnetic field of 1.6μT on the cable surface. A commonly utilised cable operating at 1600A is expected to produce an EMF of 3.2mT in a perfect wire, at the cable surface (Bochert and Zettler, 2006). As with all EMF, the values will decrease with distance from the source resulting in a field strength of 0.32mT and 0.11mT at 1m and 4m respectively (Bochert and Zettler, 2006). EMF values used in scientific studies range from 2.8 – 165mT (Formicki *et al.,* 2004; Bochert and Zettler, 2004; Woodruff *et al.,* 2012; Cada *et al.,* 2011; Scott *et al.,* 2018a). A study by Bochert and Zettler (2004) utilised a field strength of 3.7mT on blue mussel, *Mytilus edulis*, whilst another investigating rainbow trout, *Onchorhynchus mykiss*, used 1mT (Fey *et al.,* 2019). A study conducted on guppies, *Lebistes reticulatus*, used a significantly higher EMF strength of 50mT. The high variability in EMF strengths utilised in scientific studies, predicted around cables by models, and used in scientific research makes the topic of EMF research problematic.

The edible crab, *Cancer pagurus*, is a commercially important decapod found throughout western Europe from Norway to Portugal from the intertidal to depths of around 400m (Bakke *et al.,* 2019) although most commonly at depths around 100m (Neal and Wilson, 2008) (see Chapter 1.). They are heavily exploited throughout their geographic range and are the third most important shellfish fishery in the UK (Haig *et al.,* 2016), worth £44.6 million in 2014. Studies have shown that, given this species life cycle and behavioural patterns, they are highly likely to experience subsea power cables, either by previously described attraction to EMF (Scott *et al.,* 2018a) or by the creation of scour protection zones around turbine bases which subsequently act as artificial reefs (Landers Jr *et al*., 2001; Kawasaki *et al.,* 2003; Langhamer and Wilhelmsson, 2009; Linley *et al.,* 2009; Lindeboom *et al*., 2011).

The aims of the current study are to build upon the work undertaken by Scott *et al.* (2018a) and further assess the impacts of EMF exposure, at a variety of strengths predicted and observed around subsea power cables, on *C. pagurus* in order to obtain information that can be used in a dose-response model for future risk assessment.

7.2 Materials and methods

Intermoult crabs were collected from St Abbs and Eyemouth Voluntary Marine reserve (St Abbs, Berwickshire, UK) by local fishermen for experimentation. Crabs were kept in 1000 L flow through tanks with ambient sea temperature and a natural photoperiod for a minimum acclimation period of 1 week at densities of no greater than 5 crabs per tank. Crabs were sexed, weighed (g), carapace width measured (mm), and assigned a condition as per Scott *et al.* (2018a) to ensure only intact, healthy crabs were utilised. Only crabs that were at minimum landing size (≥150mm) were used during experimentation. Experimentation was conducted between May – June 2019.

Physiological analysis

Haemolymph analysis

Two $2m^3$ Helmholtz coils were utilised throughout the experiment with one set to produce a homogeneous EMF of the required strengths and the second remaining unpowered to act as a control. Building upon research conducted by Scott *et al.* (2018a) (Chapter 4. and 5.) where 2.8mT (predicted value around subsea cables) and 40mT (extreme strength to detect subtle changes in organisms) were utilised, strengths of 0.25mT, 0.5mT, and 1mT were utilised to represent the lower values predicted in specific models (Normandeau *et al.,* 2011; Hutchison *et al.,* 2018; Telford, Stevenson and MacColl Offshore Wind Farms and Transmission Infrastructure, 2019). Each Helmholtz coil was mapped using a gaussmeter (AlphaLab, Inc Gaussmeter Model GM-2) prior to experimentation.

Within each Helmholtz coil six 30 L glass tanks were set up within 60 L black ABS water baths to ensure temperature stability. Each tank contained an individual air stone, received a constant supply of temperature controlled to ambient (TECO TK2000) flow through

seawater (UV sterilised and filtered). Temperature, light intensity, and dissolved oxygen were constantly measured via data loggers (Onset HOBO temperature/light pendant) and a multiprobe (YSI ProDSS). All tanks were subjected to ambient light conditions and were covered in netting to reduce visual stimuli. Individual crabs were placed into the tanks and allowed to acclimate for a period of 1 hour. After this time baseline haemolymph samples were taken, and the coils switched on. Haemolymph samples were collected, within 60 seconds, from the arthrodial membrane at the base of the fifth walking leg using 1 ml syringes with 25G needles. 800 µl was collected from each crab and immediately transferred to a 1.5 ml cryogenic vial and frozen in liquid nitrogen. Samples were then stored in a freezer (-25°C) until use. To obtain baseline data, haemolymph was collected before exposure (0 h – 09:00am) then again after 4 h (13:00pm), 8 h (17:00pm) and 24 h (09:00am). All haemolymph collection was staggered with 5 minutes between each sample to ensure time consistency throughout the experiment. A total of 20 crabs were analysed at each field strength with 10 acting as control and 10 exposed to EMF.

Haemolymph was deproteinated using the procedure of Paterson and Spanoghe (1997). Samples were thawed, vortexed and mixed with an equal volume of chilled 0.6 M perchloric acid (BDH 10175). Inactivated proteins were separated by centrifugation at 25,000g for 10 min (Eppendorf 5417C, rotor 30 x 1.5-2ml). After neutralizing the supernatant with 3M potassium hydroxide (BDH 29628) the precipitated potassium perchlorate was separated by centrifuging at 25,000g for a further 10 min. The supernatant was then frozen and stored at -25°C.

D-Glucose

D-Glucose concentration was determined using the D-Glucose assay kit (GAGO20-1KT) as per Barrento *et al*. (2010). The stored haemolymph was thawed before analysis, 150 µl of the sample was mixed with 300 µl of reagent assay and incubated for 30 min at 37°C in a water bath. The reaction was stopped using 300 µl of 12N sulphuric acid (BDH). Absorbance was then measured in parallel measurements in microcuvettes at 540nm. D-Glucose concentrations were then calculated using a calibration curve of standards with a known concentration.

L-Lactate

Deproteinated haemolymph samples were analysed for L-Lactate concentration using colorimetric L-Lactate assay kit (Abcam ab65331). 50 µl of reaction mix (L-Lactate assay buffer (46 µl), L-Lactate substrate mix (2 µl) and L-Lactate enzyme mix (2 µl)), were added to a 50 µl deproteinated haemolymph sample. The reaction mix and sample mix were then incubated at room temperature for 30 mins then spectrophotometrically analysed in parallel measurements at 450nm. Concentrations were determined using a curve of values produced by spectrophotometrically assessing calibration standards of known concentrations.

Total Haemocyte Count

50 µl haemolymph was drawn from the pericardial sinus with sterile pre-chilled 1ml syringes (25 gauge) containing 150 µl cooled 5% (v/v) Formaldehyde (Brunel Microscope Ltd.). Haemolymph samples were dispensed to centrifuge tubes, mixed thoroughly and kept on ice to prevent coagulation. Total Haemocyte Count (THC) of individual lobsters were estimated with a Neubauer haemocytometer under magnification (X100) with a Leica (MC170 HD) compound microscope. For accuracy, samples were counted in triplicates. THC was expressed as number of cells in 1 ml of haemolymph.

Behavioural analysis

Shelter selection

Building upon the work previously conducted (Scott *et al.*, 2018a) the behavioural analysis during EMF exposure focused on shelter selection which showed a positive result during the previous study. A total of eight 70 L experimental tanks were set up with temperature controlled (13°C), flow through UV sterilised seawater. As per Scott *et al.* (2018a) one (single shelter trials) or two (dual shelter trials) black ABS shelters (300mm x 200mm x 100mm) were constructed and secured to the bottom of the tanks, with 4 solenoid electromagnets under each shelter (see Chapter 4. and 5.). The bottom and sides of each tank were covered in a neutral brown to reduce visual stimuli. During single shelter experimentation the electromagnets were switched on in 4 of the tanks with the remaining 4 acting as a control. During dual shelter trials, 4 tanks had the electromagnets

switched on under one of the shelters with other remaining switched off. The remaining 4 tanks had the electromagnets off for the duration of the trials. Tanks acting as control were randomised to reduce bias. Prior to filling the tank, the EMF was mapped for each tank using a gaussmeter (AlphaLab, Inc Gaussmeter Model GM-2) to ensure the correct field strength was obtained (0.25mT, 0.5mT, 1.0mT). An individual crab was placed into the centre of the tank and allowed to acclimate with this acclimation period being recorded. InfraRed cameras (Sannce 1080p IR surveillance DVR system) were suspended above all tanks and set to record for 24 h to ensure crab location could be confirmed, even out with experimental hours if required. The video files were then analysed from 23:00pm – 06:00am using Solomon Coder (beta version 17.03.22) to determine the percentage of time spent in the shelters or free roaming within the tank.

Statistical analysis

Results were expressed as mean ± standard error (SEM). Data was assessed for normality using Shapiro-Wilk test for normality and Levene's test for equality of error variances. When data met these assumptions, repeated measures multivariate analysis of variance (MANOVA) followed by post-hoc analysis by Tukey's test was used. Differences between the treatments were tested by Student's t-test and paired samples t-test as appropriate. If data did not meet parametric assumptions Mann-Whitney U-test and Wilcoxon matched pairs signed rank test were used. All statistics were tested at a probability of 0.05 (IBM SPSS Statistics v.23 SPSS Corp. Chicago, USA).

7.3 Results

Physiological analysis

Haemolymph analysis

D-Glucose levels followed a similar circadian rhythm in control and EMF exposed crabs, with significant increases towards peak locomotor activity ($F(3,316)=59.98$, $p<0.05$, repeated measures ANOVA). D-Glucose concentrations showed significant increases between 0 h and 4 h and 0 h and 8 h in control conditions and during exposure to all three EMF strengths ($p<0.05$, posthoc Tukey's test) (Figure 7.1.). D-Glucose concentrations

had returned to initial levels after 24 h in all samples, resulting in no significant difference from 0h. There were no significant differences in D-Glucose concentration between control and 0.25mT EMF exposed crabs at any sampling point ($F(3,376)=0.87 p>0.05$, repeated measures MANOVA) with mean values throughout the 24h sampling period remaining relatively low in comparison to the other EMF strengths (control 0.462±0.03 mM, 0.25mT 0.445±0.05 mM). Crabs exposed to 0.5mT and 1mT EMF were showing signs of hyperglycaemia after 4h (0.914±0.11mM, 1.063±0.11 mM respectively) and 8 h (0.889±0.11 mM, 0.973±0.11 mM respectively) exposure compared to the control group (0.6±0.06 mM, 0.551±0.07 mM), ($p<0.05$, posthoc Tukey's test). Haemolymph D-Glucose concentrations after 24 h exposure to 0.5mT and 1mT EMF returned to baseline levels and did not differ significantly from the control value at 24 h. Significant differences in D-Glucose concentration were observed between sampling times and between treatments ($F(3,316)=17.51$, $p<0.001$, $F(3,316)=4.12$ $p<0.05$, repeated measures MANOVA).

L-Lactate followed a circadian rhythm with increased concentrations coinciding with periods of high activity in control conditions and during exposure to 0.25mT EMF (8h, 0.625±0.12 mM and 0.757±0.22 mM, for control and 0.25mT respectively) ($F(3,76)=3.6$, $p<0.05$, repeated measures ANOVA). L-Lactate concentrations in 0.5mT and 1mT exposed crabs did not show signs of following a circadian rhythm with no significant increases leading up to the periods of increased activity (Figure 7.2.). Significant differences were observed in L-Lactate concentrations among treatments at different sampling times ($F(3,316)=2.92$, $p<0.05$, repeated measures ANOVA). Crabs exposed to 1mT EMF showed significantly lower L-Lactate concentrations throughout the 24h period (0.195±0.12 mM, $p<0.05$, posthoc Tukey's test), when compared to the control values. Crabs exposed to 0.25mT EMF had significantly lower L-Lactate concentrations after 4h exposure (0.194±0.1 mM, $p<0.05$, posthoc Tukey's test), while 0.5mT exposed crabs showed significantly lower concentrations after 24 h exposure (0.230±0.03 mM, $p<0.05$, posthoc Tukey's test) when compared to the control group (4 h, 0.482±0.06 mM, 8 h, 0.5111±0.06 mM).

Average THC in control crabs was 28.64±2.2 x 10^6 cell ml^{-1}. Short-term rhythmical fluctuations in control crab's THC over the 24 h sampling period was noted, with significantly lower values after 24 h (Figure 7.3.) ($F(3,156)=3.82$, $p<0.05$, repeated

measures ANOVA). THC of crabs exposed to 0.25mT EMF did not differ significantly from values found in crabs kept in control condition, with similar fluctuations during the 24 h period. THC of crabs exposed to 0.5mT and 1mT EMF did not show similar fluctuations with no significant decrease after 24 h of exposure. Crabs exposed to 0.5mT and 1mT showed slightly elevated values (38.73±6.4 and 36.33±6.4 2 x 10^6 cell ml^{-1} respectively) after 8h of exposure when compared to control values (22.51±.5 x 10^6 cell ml^{-1}), but was only statistically significant at 0.5mT strength ($p<0.05$, posthoc Tukey's test). THC values after 24 h exposure to 0.5mT and 1mT EMF returned to basal values and no statistically significant differences were found between experimental and control groups.

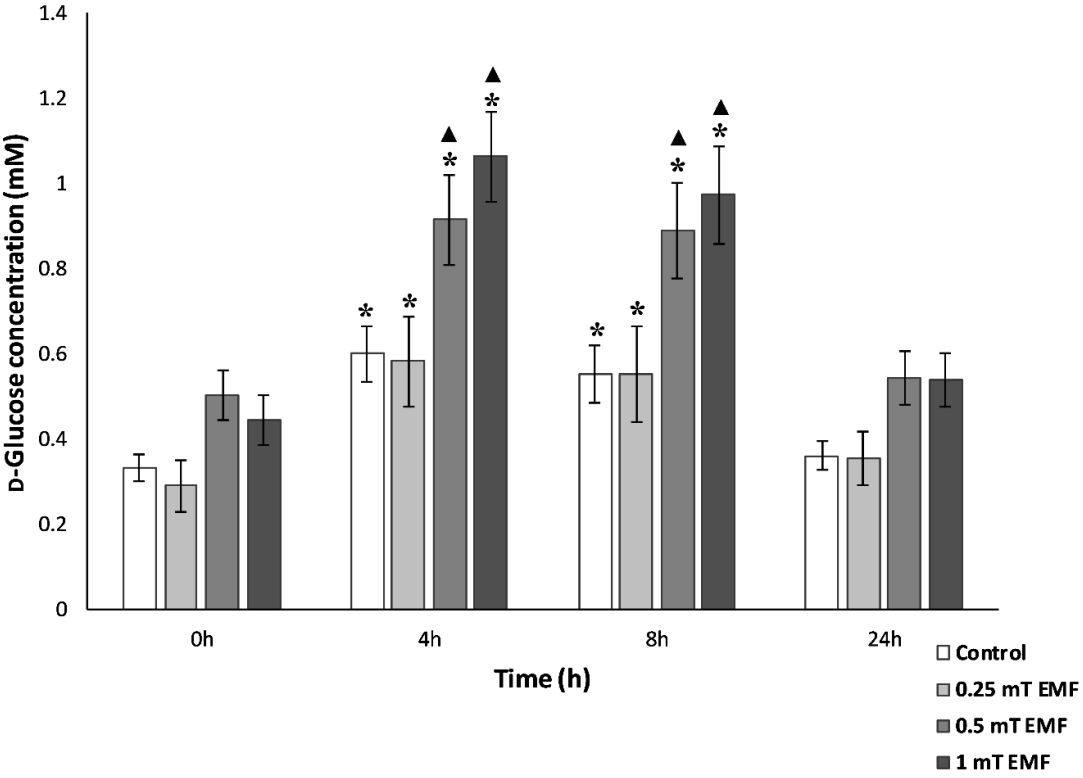


Figure 7.1. D-Glucose concentration over a 24 h period in control conditions and exposure to EMF at 0.25mT, 0.5mT, 1mT strength. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). Values are presented as Mean ± SEM, *is the significance from the 0 h within respective treatments, ▲ is the significance from the control group for each treatment at respective sampling times (*, $p<0.05$), (▲, $p<0.05$). N=10 for control and N=10 for EMF per treatment, total N=40 control, N=40 EMF.

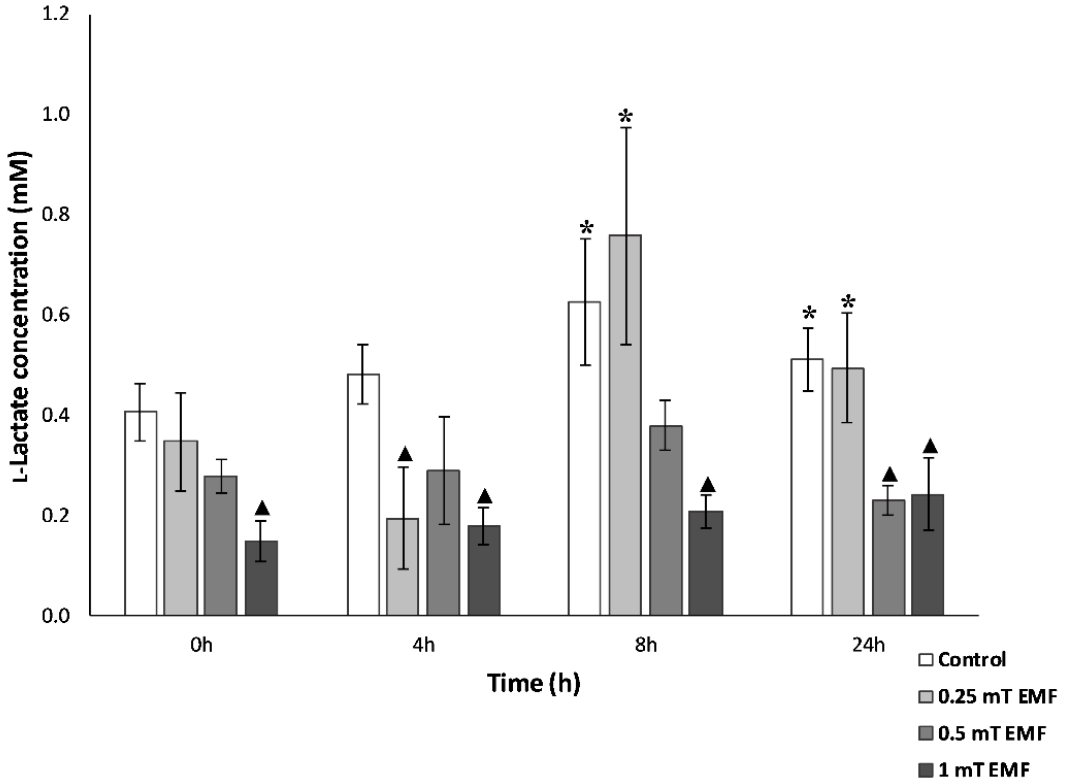


Figure 7.2. L-Lactate concentration over a 24 h period in control conditions and exposure to EMF at 0.25mT, 0.5mT, 1mT strength. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). Values are presented as Mean ± SEM, *is the significance from the 0 h within respective treatments, ▲ is the significance from the control group for each treatment at respective sampling times (*, $p<0.05$), (▲, $p<0.05$). N=10 for control and N=10 for EMF per treatment, total N=40 control, N=40 EMF.

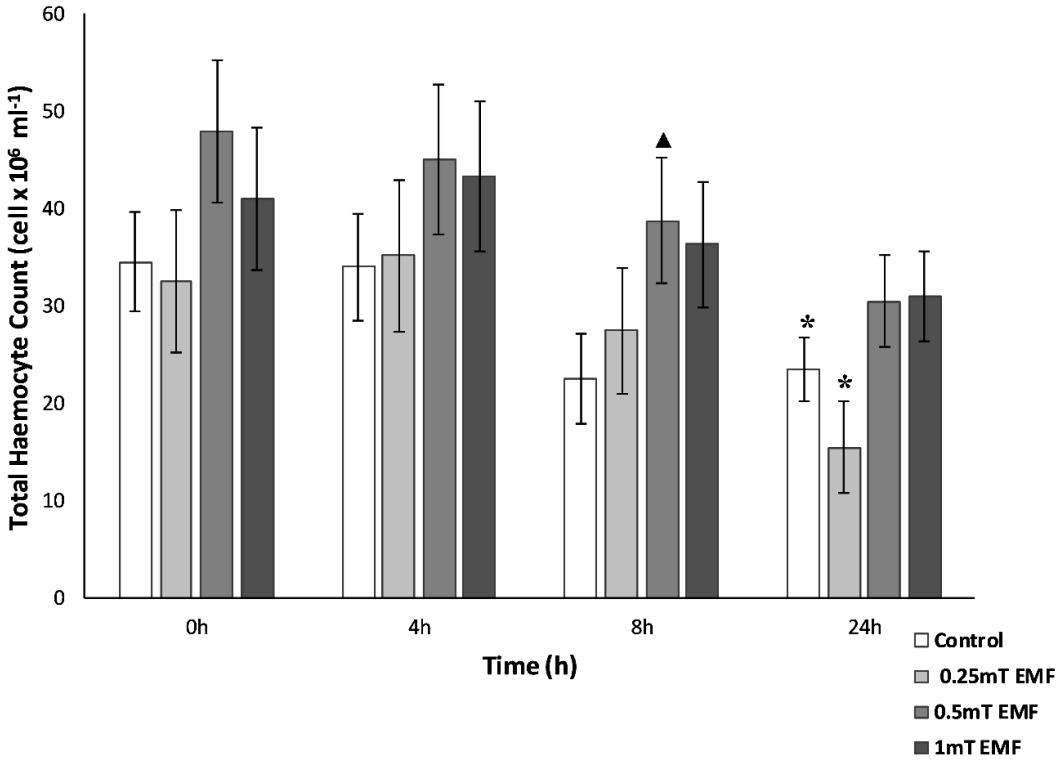


Figure 7.3. Total Haemocyte Count (THC) over a 24 h period in control conditions and constant exposure to static EMF at 0.25mT, 0.5mT, 1mT strength. Sample times consisted of 0 h (09:00am), 4 h (13:00pm), 8 h (17:00pm), 24 h (09:00am). Values are presented as Mean ± SEM, *is the significance from the 0 h within respective treatments, ▲ is the significance from the control group for each treatment at respective sampling times (*, $p<0.05$), (▲, $p<0.05$). N=10 for control and N=10 for EMF per treatment, total N=40 control, N=40 EMF.

Behavioural analysis

Single shelter preference

The mean time spent in the shelter (256.20±36.6 min) was slightly higher than time spent roaming the tank (163.80±36.6 min) in control trials (Figure 7.4.). When there was an EMF of 0.25mT present there were no significant differences between the time spent in (273.50±14.9 min) and out (146.50±14.9 min) of the shelter compared to the control. A similar pattern was observed during exposure to 0.5mT with no significant differences

being found between time spent in (222.63±49.1 min) and out (197.38±49.1 min) of the shelter, despite a slight increase in time spent roaming the tank. Crabs spent significantly more time in the shelter (319.63±25.7 min) during exposure to 1mT EMF ($F(1,18)=36.3$, $p<0.001$, one-way ANOVA).

Dual shelter preference

Under control conditions a near equal split of time was spent between the EMF shelter (169.3±9.6 min) and the control shelter (172±12.4 min) with 79.8±7.2 min time spent roaming the tank (Figure 7.4.). Exposure to 0.25mT EMF did not produce any significant changes in the time spent in shelters or roaming the tank (58.8±10.2 min No shelter, 155.4±11.6 min EMF shelter, 205.8±13 min control shelter). Exposure to both 0.5mT and 1mT showed significant differences compared to the control ($F(3,16)=13.2$, $p<0.001$, $F(3,16)=24.3$, $p<0.001$, MANOVA), with an increased amount of time spent within the EMF shelter (264.6±11 min, and 286.5±9.8 min for 0.5mT and 1mT respectively). There was a drop in the mean time spent roaming the tank from 79.8±7.2 min in control conditions to 42±6.4 min and 28.5±3.5 min during exposure to 0.5mT and 1mT EMF respectively.

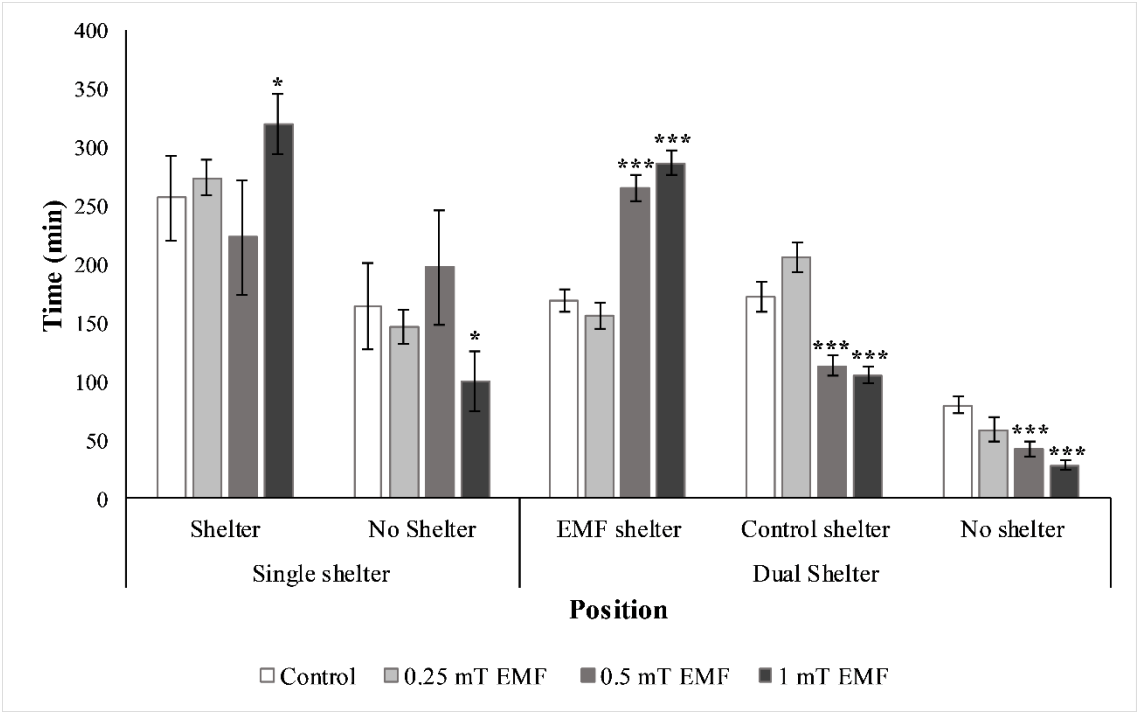


Figure 7.4. The effects of EMF exposure at various strengths on shelter selection in *C. pagurus*. Mean time spent in the shelter and roaming the tank in control conditions and exposure to 0.25mT, 05mT, and 1mT EMF in single shelter trials. Mean time spent within control and EMF exposed (0.25mT, 05mT, and 1mT EMF) shelter and roaming the tank in dual shelter trials. Calculated as time spent in each location compared to total trial length. Values are presented as Mean ± SEM, * is the significance at $p<0.05$, ** is the significance at $p<0.01$, *** is the significance at $p<0.001$. Single shelter $N_{CONTROL} = 10$, $N_{EMF} = 10$, dual shelter $N_{CONTROL} = 10$, $N_{EMF} = 10$.

7.4 Discussion

L-Lactate and D-Glucose concentrations followed a natural circadian rhythm with rises in D-Glucose throughout the day and an increase in L-Lactate in the evening corresponding with periods of higher activity. This circadian rhythm corresponds well to that found in the literature (Scott *et al.*, 2018a). Values obtained for both L-Lactate (Watt *et al.*, 1999; Lorenzon *et al.*, 2008; Barrento *et al.*, 2009) and D-Glucose (Reddy *et al.*, 1981; Kallen *et al.*, 1990; Tilden *et al.*, 2001; Scott *et al.*, 2018a) correspond to those found in previous studies. L-Lactate concentrations observed during this study were lower than those values

recorded for *C. pagurus* in Chapter 2. and 4. however still followed the same diel pattern. This variation in values could be due to the use of a different assay kit during analysis or due to the high individual variation found within this species (Scott *et al.,* 2018a). As described in Chapters 2. and 4. L-Lactate levels rise during periods of high locomotor activity resulting in an increased glucose metabolism. At the same time, D-Glucose levels decrease due to the increased oxidation for ATP production (Barrento *et al.,* 2010). The results obtained throughout this study suggest that exposure to 0.25mT EMF does not alter the circadian rhythm of L-Lactate and D-Glucose. Exposure to EMF at 0.5mT and 1mT showed the same results as described in the literature during exposure to 2.8mT (Scott *et al.,* 2018a) whereby L-Lactate fails to show the expected increase during periods of higher activity. The suppression of L-Lactate will impact the O_2 affinity of Haemocyanin, which has been shown to increase during periods of high L-Lactate concentrations (Sanders and Childress, 1992) to allow more oxygen to be transported around the body to counteract periods of hypoxia. Exposure to 0.5mT and 1mT EMF elicits the same responses in D-Glucose as has been previously described in this species during exposure to a field strength of 2.8mT (Scott *et al.,* 2018a). Despite following the same circadian rhythm, D-Glucose concentrations were significantly higher after 4 h and 8 h before returning to normal levels after 24 h. Haemolymph D-Glucose and L-Lactate should cycle together in normal unstressed conditions (Scott *et al.,* 2018a) but have been shown to be affected by environmental stressors (Kallen *et al.,* 1990; Reddy *et al.,* 1996; Chang *et al.,* 1998). D-Glucose has been shown to have a negative correlation with vigour, with moribund crabs becoming hyperglycaemic (Barrento *et al.,* 2010). Evidence suggests that D-Glucose and L-Lactate cycles are controlled by melatonin, a neuropeptide present in crustaceans (Tilden *et al.,* 2001). Earlier studies have suggested that EMF exposure impacts melatonin levels (Reiter, 1993; Schneider *et al.,* 1994; Levine *et al.,* 1995; Wood *et al.,* 1998). This study adds more evidence to this hypothesis by finding similar circadian disruption in D-Glucose and L-Lactate at 0.5mT and 1mT EMF exposure.

The THC values obtained throughout this study correspond well with those previously recorded for *C. pagurus* in the literature. Vogan and Rowley, (1990) recorded values of $2.55\pm0.14x10^7$ cells/ml, Lorenzon *et al.* (2008) found values of $3.19\pm0.92\times10^7$ cell/ml and more recently Parrinello *et al.* (2015) observed values of $4.4 \pm 0.6x10^7$ cell/ml in *C. pagurus*. In the shore crab, *Carcinus maenas*, a study conducted in North Wales by

Truscott and White, (1990) found significant differences in THC concentrations between high and low tides with double the concentration recorded at 8m compared to a 4m tide.

In Chapter 5. THC in *H. gammarus* was significantly affected by exposure to an EMF of 2.8mT resulting in lower mean values after 12 h with significant increases between 6 h and 24 h. However, in this study a significant rise after 8h was detected during exposure to 0.5mT EMF. During exposure to control and 0.25mT there were significant drops in THC after 24 h, whilst no significant decreases were detected in 0.5mT and 1mT. Large variations in THC concentration was found in individuals throughout this study which may have masked some of the effects of the treatment. Previous studies have shown that THC levels rise during exposure to increased stress (Le Moullac and Haffner, 2000; Monari *et al.,* 2007) suggesting an immune response. However, the reverse has also been confirmed with a decrease in THC resulting from the presence of stressors including bacteria (Stewart, 1967), hypoxia (Le Moullac *et al.,* 1998) and EMF (Valadez-Lira *et al.,* 2017). Significant variations from the normal rhythmic patterns of THC were detected during exposure to 0.5mT and 1mT suggesting the beginning of an immune response, Exposure to 0.25mT showed no significant differences from the control suggesting low strength EMF may not result in reduced immune capacity.

During single shelter trials a higher percentage of time was spent within the shelter than out roaming the tank, although this was lower than the results obtained for *C. pagurus* in Chapter 4. This conforms to previous findings that crustaceans utilise shelters (Skajaa *et al.*, 1998) with periods of time spent roaming out with (Scott *et al.*, 2018a). No significant changes were noted when the shelter was subjected to an EMF of 0.25mT. This result combined with those obtained from the physiological analysis suggests that EMF exposure of 0.25mT does not negatively impact *C. pagurus* on a behavioural or physiological level like has previously been found with higher strengths (Scott *et al.,* 2018a). During exposure to 0.5mT EMF, a slight decrease in time spent in the shelter occurred, which also occurred with *H. gammarus* in Chapter 5. At 1mT there was a clear attraction to the source of the EMF with a significant increase in the time spent within the EMF exposed shelter. Results obtained from the dual shelter trials confirm an apparent lack of response during exposure to 0.25mT. The remaining EMF strengths highlighted an attraction for the EMF source with significant increases in the time spent within the shelter. This has clear implications as previously discussed in Chapters 4. and 5., whereby

crabs show increased attraction to the source of the EMF, despite showing signs of physiological stress. An attraction to subsea power cables emitting an EMF of 0.5mT+, which appears to come at the cost of time spent foraging for food, seeking mates and potentially finding shelter, is a cause for concern. With the addition of scour protection zones aiming to increase biodiversity (Kawasaki *et al.,* 2003; Linley *et al.,* 2009), and the plan to utilise offshore renewable structures for aquaculture (Holm *et al.,* 2017; Causon and Gill, 2018), there is a clear need to consider EMF emissions more closely.

The results obtained from this study, combined with the data from Chapter 4., suggests that increased physiological stress will occur if *C. pagurus* is exposed to EMF of 0.5mT or more with data obtained at 1mT, 2.8mT and 40mT confirming this trend. This suggests that a working limit of 0.25mT would result in minimal physiological changes within this species and should be considered during MRED design and implementation.

Chapter 8. General discussion and conclusions

The overarching aim of this project was to contribute to the lack of data on the effects of EMF exposure on two commercially and ecologically important crustaceans, *Homarus gammarus* and *Cancer pagurus*. In the literature both species, despite their importance, have to date been eclipsed by their American counterparts (*H. americanus* and *Metacarcinus* (previously *Cancer magister*) in terms of research outputs, particularly in relation to Marine Renewable Energy Devices (Woodruff *et al.,* 2012, 2013; Love *et al.,* 2015; Hutchison *et al.,* 2018). Whilst there are many similarities between the biology of the respective species pairs (*H. gammarus* and *H. americanus*; *C. pagurus* and *M. magister*), there are also differences such that we cannot assume all results in one species will be directly transferrable to the other. Throughout this study several original observations were made for both *C. pagurus* and *H. gammarus* which extend our understanding of key elements in the ecology of both. These are discussion below.

Cancer pagurus

Activity level in *C. pagurus* was found to be size linked with smaller crabs having higher activity levels, whilst larger mature individuals were more sedentary and appeared to sense their environment more through increased antennular flicking than by direct exploration. Juvenile crabs, which inhabit high biomass intertidal areas such as kelp forests (Mann, 1973; Ricklefs and Miller, 2000; Park, 2001) are likely to be subjected to greater rates of predation from birds, cod, seals, wolffish, and octopus (Rae 1967, 1968; Rae and Shelton, 1982; Boyle *et al.,* 1986; Skajaa *et al.,* 1998; Tallack, 2002), and competition for resources like food, and shelter due to the higher number of organisms inhabiting these areas. With higher densities of individuals potentially leading to resource depletion around shelters, this may result in the need to relocate, which may problematic due to the apparent bottleneck of shelters in the wild (Wahle and Steneck, 1991). Shelter utilisation is common in crustaceans (Chapman and Rice, 1971; Hockett and Kritzler, 1972; Hazeltt and Rittschof, 1975; Hill, 1976) with *C. pagurus* utilising a variety of shelters including rocks, crevices, pits dug in the substrate (Hall *et al.,* 1991) and the recently confirmed holdfasts of *L. digitata* and *L. hyperborea* (Scott *et al.,* submitted). Larger crabs tend to inhabit deeper waters where the pressures experienced by the

juveniles will not be as pronounced due to an overall decrease in biomass. With decreased competition for shelter, food, and predation, which has been shown to decrease with larger sized individuals (Paine, 1976) the larger crabs can afford to be less mobile and rely on sensory organs such as antennules for chemo-sensing their environment. Whilst larger crabs tend to show lower activity levels, they do undertake seasonal migrations which juveniles are not known to perform. In a study by Ungfors *et al.* (2007) differences in movement were found between individual adult males with some remaining in the same area for weeks at a time whilst others made regular movements throughout the sample period.

In relation to diel activity patterns, *C. pagurus* has generally been classed as nocturnal (Skajaa *et al.*, 1998), and this was partially confirmed by this study. Activity levels were found to be higher at night compared to daytime hours, although it was noted that crabs tended to exhibit a more crepuscular, rather than strictly nocturnal, behaviour with peak activity occurring around and dawn and dusk. The present study also confirmed for the first time that juveniles, whilst showing different activity levels to adults, also exhibit crepuscular and nocturnal behaviour. Foraging mostly occurs at night during these periods of high activity and has likely evolved to reduce risk of predation by the visual predators previously mentioned, whilst also serving to enhance the food spectrum, by way of preying on faster diurnal species at rest and pursuit success akin to *Carcinus maenas* (Naylor, 1960; McDonald *et al.*, 2001). Whilst there are obvious behavioural differences in male and female *C. pagurus* in regard to migration patterns (Karlsson and Christiansen, 1996; Ungfors *et al.*, 2007) and aggressive interactions (Warner and Warner, 1977; Edwards, 1979) there was found to be no difference in activity level and antennular flicking rates between sexes. During periods leading up to copulation, several species of crabs have been shown to utilise pheromones to attract mates including *Telmessus cheiragonus* (Kamio *et al.*, 2000) and *Carcinus maeanas* (Eales, 1973). If pheromones were utilised in *C. pagurus* during mate attraction, this could result in seasonal differences in antennular flicking rates between male and females that were not detected during these studies. Further studies on the potential for copulation pheromones, their detection methods, and potential anthropogenic process that may have an impact on detection is required. Sex linked differences were found in several components throughout this study.

This study also addressed some much-needed information on the basal conditions of physiological parameters commonly used for assessing impacts of marine stressors. The present study is the first to reveal that a circadian rhythmic cycle exists for both L-Lactate and D-Glucose concentrations in the haemolymph of *C. pagurus*. D-Glucose, the primary fuel for ATP production in crustaceans (Barrento *et al.,* 2010), was found to rise throughout the day with a simultaneous decrease in L-Lactate, a metabolite commonly indicating anaerobic respiration (Durand *et al.,* 2000). D-Glucose concentrations were shown to continually rise in relation to increased locomotor activity (Hamann, 1974; Kallen, *et al.,* 1988; Kallen *et al.,* 1990; Reddy *et al.,* 1981; Tilden *et al.,* 2001) with activity level reflected in D-Glucose concentrations (Briffa and Elwood, 2001). D-Glucose shows high variability amongst individuals due to differences in individual crab physiology and reactions to external stimuli (Matsumasa and Murai, 2005). L-Lactate was found to be highly variable between individuals as well as being size-dependent, with smaller crabs having lower concentrations than larger crabs. However, there were no differences found between males and females. Values obtained throughout this study for D-Glucose and L-Lactate corresponded well to the range of values obtained in the literature (Watt *et al.,* 1999; Lorenzon *et al.,* 2008; Barrento *et al.,* 2010; Barrento *et al.,* 2011) but expanded upon this knowledge by examining temporal changes over a 24h period. These diel circadian changes in concentrations of both L-Lactate and D-Glucose should be incorporated into future studies on this species to accurately determine physiological changes. Haemocyanin, the primary oxygen carrying protein in crustaceans (Hagerman *et al.,* 1990), was found not to be size or sexed linked and does not follow a circadian rhythm. Haemocyanin levels have previously been documented rising during period of hypoxia (Hagerman *et al.,* 1990), and has been found to be influenced by the concentration of L-Lactate which increases the O_2 affinity of Haemocyanin during high concentrations (Sanders and Childress, 1992). Previous studies have shown that *C. pagurus* exhibit high respiratory independence (Bradford and Taylor, 1982) obtained through alternating periods of apnoea and bradychardia in the branchial chambers (Bottoms; Burnett and Bridges, 1981). As such obtaining accurate values can be difficult, however there is a lack of data in the literature on the respiration rates of juveniles. This study, although limited by respiration chamber size, was able to obtain respiration values for juveniles that were comparable to similar sized crabs including *Carcinus maenas*, *Necora puber*, *Metacarcinus magister*, and *Hyas araneus* (Johansen, 1970; Newell *et al.*,

1972; Taylor and Butler, 1978; Taylor and Wheatly, 1979; Camus *et al.*, 2002; Small *et al.*, 2010). Respiration was found to be significantly higher in juvenile female crabs than males which is the first recording for this species to the authors knowledge however, this has previously been noted in the blue crab, *Callinectes sapidus*, (Engel and Eggert, 1974). Several other studies have shown in some species there are no sex-linked differences in respiration (Valiela *et al.*, 1974) or female respiration is lower than males (Dimock and Groves, 1975). In Fiddler crabs, *Uca pugilator*, respiration rates were significantly higher in male crabs due to the increased metabolic costs of having large claws, used for defense and mating displays (Allen and Levinton, 2007).

Increased respiration in females may result in increased food requirements, which may partially explain the lack of juvenile females found in the intertidal zones. Juvenile females may need to extend their foraging radius in to the subtidal to increase the variety of prey available. Food availability has been shown to be more abundant in subtidal zones than intertidal where increased wind and wave action has a negative impact (Smaal and Haas, 1997).

Exposure to EMF, at the strength predicted around subsea power cables (2.8mT), was found to have no impact on several key parameters including Haemoycanin, respiration, activity level and antennular flicking rates, this analysis being facilitated by the provision of the extensive baseline data mentioned above which were not previously available. This highlights the need more broadly for detailed information on stress biomarker baselines in a wide range of species not yet studied in detail, in order to underpin effective risk assessment of novel human activities in the marine environment. Furthermore, significant disruption was found in the L-Lactate and D-Glucose circadian rhythms. Under normal unstressed conditions L-Lactate and D-Glucose should cycle inversely together, however during periods of physiological stress previous literature has shown significant deviations in concentrations (Kallen *et al.*, 1990; Reddy *et al.*, 1996; Chang *et al.*, 1998). Melatonin, a neuropeptide present in crustaceans, can cause shift in L-Lactate and D-Glucose cycles (Tilden *et al.*, 2001) and has been shown to be affected by exposure to EMF (Reiter, 1993; Schneider *et al.*, 1994; Levine *et al.*, 1995; Wood *et al.*, 1998). During exposure to an EMF of 2.8mT L-Lactate concentration followed the predicted pattern of decreasing throughout the day, however failed to show elevated levels after increased activity during the night. With previous studies showing that L-Lactate is

essential in kickstarting the processes involved in repaying oxygen debts accrued during periods of high activity, there is a possibility that crabs exposed to EMF around subsea power cables will be unable to regulate oxygen as efficiently throughout their bodies potentially limiting metabolic scope. Despite no significant changes in activity levels during exposure to EMF, D-Glucose concentrations were significantly altered. D-Glucose concentrations appeared to follow the predicted significant upward curve for the initial 4 h during the daytime then showed no discernible pattern thereafter. The lack of significant changes in activity levels, known to be linked to D-Glucose concentrations, suggests that the cycles are being altered via internal processes, most possibly melatonin. Further studies are needed to determine the effects of EMF exposure on melatonin levels in *C. pagurus* to fully assess the processes behind this apparent physiological disruption. D-Glucose concentrations confirmed an altered cycle by following the same predicted patterns but showing higher overall concentrations at 4 h and 8 h. These physiological changes stimulated by exposure to increased anthropogenic EMF suggests that this species cannot maintain homeostasis during exposure. Further research needs to be conducted to determine the long-term effects of chronic EMF exposure on physiological processes within this species.

Behavioural changes were also recorded during exposure to EMF. During exposure crabs were unable to find a suitable place to rest, however overall activity levels did not accurately reflect these changes. As previously mentioned, shelter utilisation is important in crustaceans and this may partially explain the somewhat conflicting results. When shelter was made available, crabs consistently chose to utilise it regardless of EMF presence. When two shelters were made available, one exposed to EMF and the other acting as a control, the crabs continued to show attraction to the EMF shelter. This attraction was strong enough to significantly reduce the time spent roaming the tank. Given the hunting repertoire associated with this species of crab (described by Lawton and Hughes, 1985), which is characterised by preying upon slow moving invertebrates, a significant amount of time foraging across the benthic environment can be expected in order to consume sufficient food. The discovery that EMF exposure reduces the amount of time spent foraging is a potential cause for concern in relation to disruption of normal foraging patterns and the possibility of compromised, however research needs to be expanded into the field to fully assess changes in foraging behaviour in EMF exposed areas. Given the physiological stress that exposure to increased EMF elicits, combined

with the apparent attraction to the EMF source, the benefits associated with creating no take zones around MREDs could become unsuccessful due to the potential reduction in viable larvae released from ovigerous females brooding near cables. The increase in biomass associated with the addition of scour protection zones acting as artificial reefs (Bortone *et al.*, 1994; Kawasaki *et al.*, 2003; Langhamer and Wilhelmsson, 2009) and the predicted spill-over effect of species into surrounding fished areas could also be negatively impacted by the attraction to EMF sources. EMF exposure negatively impacted the development of eggs, with significant changes in egg volume detected throughout development. Upon hatching, zoea were significantly smaller across a broad range of measurements, however larval locomotor performance was not impacted by development occurring under EMF exposure. Whilst many factors remained unchanged, the differences in larval morphology and egg volume detected during EMF exposure are a cause for concern which merits further detailed study. Given the high likelihood of brooding females coming in to contact with subsea power cables and the recently proven attraction *C. pagurus* shows to the emitted EMF, there is likely to be a significant number of females who will brood eggs within an increased EMF. The impacts of this on stock recruitment and population dynamics must be studied to fully understand the impacts of EMF on reproductive success.

Exposure to an EMF of 0.25mT did not seem to induce physiological stress in any of the measured parameters in *C. pagurus* whilst the remaining values assessed (0.5mT, 1mT, 2.8mT and 40mT) affected Total Haemocyte Counts (THC), suggesting an immune response, and the circadian rhythms of D-Glucose and L-Lactate, in addition to inciting behavioural changes. Given the large variation in predicted EMF strengths around subsea power cables, this data provides a starting point in assessing 'safe' exposure limits that can utilised to minimise disruption to this species.

Homarus gammarus

A considerable amount of information exists on adult lobsters, both the European lobster, *H. gammarus*, and the closely related *H. americanus*, however very little is known about the ecology of larvae and juveniles (Howard and Bennett, 1979). Juvenile lobsters are rarely caught in baited traps, rarely observed in the plankton, and previous scientific

surveys using divers have found a limited number of juveniles (Howard and Bennett, 1979). Juveniles have been shown to dig complex burrows in fine particulate substrates thus differing from adult behaviour (Howard and Bennett, 1979). Due to the difficulty in obtaining wild juvenile *H. gammarus*, the majority of the experiments utilised throughout this study utilised different size classifications for landed lobsters caught in baited traps. Despite many studies on *H. gammarus*, there is a lack of baseline data on many key physiological and behavioural parameters, and how they may vary between different size and sex.

H. gammarus, like many other crustaceans are generally considered to be nocturnal, however this study highlights a crepuscular behaviour with increased movement occurring within the first few hours after sunset, which has been observed in several other studies (Smith *et al.,* 1998, 1999). There is high degree of individual variability in the activity and movement levels of *H. gammarus*, which may be partly explained by different 'personalities' which have been observed in other decapod crustaceans (Gherardi *et al.,* 2012). Despite a recent surge in scientific interest of animal behaviour, very few studies have attempted to investigate the issue of behavioural plasticity in crustacean decapods (Gherardi *et al.,* 2012), although several studies have shown that individual variation in marine invertebrates does exist (Gosling, 2001; Sinn and Moltschaniwskyj, 2005; Sinn *et al.,* 2006; Briffa and Greenaway, 2011). For example, *H. americanus* have been shown to alternate nocturnal and diurnal behaviour with greater periods of activity occurring throughout the day (Golet *et al.,* 2006). Seasonality is known to have an effect on the activity level of *H. gammarus*, with a reduction in activity during the winter, likely due to decreased metabolism (Smith *et al.,* 1998). Activity levels have previously been found to be size linked, with smaller individuals exhibiting higher activity levels (Mehrtens *et al.,* 2005). As with *C. pagurus* juveniles, the observed increase in activity levels may correspond to the different environmental pressures that are subsequently reduced as the animals grows including competition for food, shelter, increased predation etc (Chapman and Rice, 1971; Hockett and Kritzler, 1972; Hazeltt and Rittschof, 1975; Hill, 1976; Paine, 1976). Haemolymph density was found to be the only parameter in this study that significantly differed between males and females with male lobsters containing higher mean values which appears to be the first recorded case in *H. gammarus*. As was found in *C. pagurus*, Haemocyanin concentrations did not show any circadian or size linked patterns and remains at a constant concentration, presumably

only altering during periods of oxidative stress (Hagerman *et al.,* 1990). However, there was a near significance variation between males and females. Previous studies have shown that in some species significant variations occur between sex, with males found to contain higher levels (Horn and Kerr, 1969; Bellelli *et al.,* 1988).

Total Haemocyte Counts obtained within this study show high individual variability, with no discernible patterns between sexes or sizes. The high variability found throughout *H. gammarus*, combined with higher mean values compared to other crustaceans (Jussila *et al.,* 1997, 2001; Hernroth *et al.,* 2004; Celi *et al.,* 2014; Simon *et al.,* 2016; Powell *et al.,* 2017; Ooi *et al.,* 2019) highlights the need for species-specific measurements of factors previously thought of as relatively constant across several crustacean species.

D-Glucose and L-Lactate did appear to show slight deviations throughout the 24h sample period suggesting a circadian rhythm is present in *H. gammarus*. However, the results were not as clear as those obtained for *C. pagurus*. D-Glucose was shown to rise throughout the day, however due to differences in peak activity between individuals the overall trend was masked. L-Lactate did show a slight decrease throughout the day, although not significant, does follow the predicted patterns of a diel rhythm. The circadian rhythm thought to occur in *H. gammarus* could follow a longer timescale than 24 h which would have been missed throughout the sampling in this study. High variability within individuals resulting in slightly obscured mean values could also have attributed to the lack of identifying a clear D-Glucose and L-Lactate circadian rhythm. Crustaceans also have a complex physiology with multiple processes that may overlap and influence each other (e.g. Aréchiga *et al.,* 1993; Fanjul-Moles, 2006).

Seasonal changes within *H. gammarus* warrants further investigation beyond the changes in Haemocyanin concentration (Bellelli *et al.,* 1988) and activity levels (Smith *et al.,* 1998) previously recorded. The results from this study highlight that individual variability must be factored in when assessing changes in the behaviour and physiology of this species in future studies.

An attraction to EMF, at 2.8mT, was detected in *H. gammarus* although activity levels remained unaffected. Despite high individual variability in activity levels found within the baseline study and in previous literature (Golet *et al.,* 2006), little variation was found during the EMF trials. Lobsters utilised the shelter during the single shelter trials for 90%

of the study time, despite coinciding with predicted periods of highest activity (Golet *et al.*, 2006). Shelter is an important and sometimes limiting factor in benthic crustaceans (Stein and Magnuson, 1976; Bertness 1981; Yoshimura and Yamakawa, 1988), particularly for lobster (Caddy 1986; Fogarty and Idoine 1986). The dual shelter trials highlighted a significant attraction to the source of the emitted EMF, with almost unanimous selection of the EMF exposed shelter by the lobsters throughout the trials. These results echo those found with *C. pagurus* and suggest that, not only is there detection of the EMF but an active attraction to it. The results obtained from this study, combined with the knowledge that previously disturbed lobsters will seek shelter quicker (Van der Meeren, 2002), possibly suggest that the lobsters used during this study were either suffering from lingering handling stress, stress induced by EMF exposure, or were displaying an increased curiosity reflex (Formicki *et al.*, 2004). Lobsters were acclimated for a period of at least 1 hour prior to experimentation which is considerably longer than acclimation periods utilised in previous lobster behavioural studies (Karavanich and Atema, 1998). An increased curiosity reflex has been observed in fish during exposure to EMF, which may partially influence the selection of the EMF exposed shelter.

Significant physiological effects were found during exposure to EMF with deviations in several key haemolymph parameters including THC, D-Glucose and L-Lactate concentrations. D-Glucose concentrations significantly increased over the 24 h during exposure. High levels of D-Glucose have been found in moribund crustaceans, and subsequently lower values found in healthy individuals (Barrento *et al.*, 2010). This suggests that lobsters are showing the onset of hyperglycaemia and subsequently confirming a state of increased physiological stress brought on by exposure to EMF. Regulating D-Glucose concentrations is essential to maintaining the brain and muscles. The subsequent inability to regulate D-Glucose may lead to significant decrease in vigour (Barrento *et al.*, 2010) and inability to produce ATP at a sufficient rate (Barrento *et al.*, 2010). A significant increase in L-Lactate in lobster haemolymph occurred after 12h of EMF exposure. This suggests a higher degree of anaerobic respiration occurs after the onset of EMF exposure, with concentrations returning to those comparable with control after 24 h. Increased L-Lactate has been associated with increasing the O_2 affinity of Haemocyanin (Sanders and Childress, 1992) to aid in periods of hypoxia. In *Carcinus maenas* L-Lactate concentrations significantly rose during periods of emersion and remained elevated for 1h after immersion which suggests the need for the organism to

repay an oxygen debt (Simonik and Henry, 2014). This oxygen debt could partially explain the extended response in *H. gammarus* after EMF exposure and the subsequent decrease to baseline concentrations after 12 h. The changes that occurred in L-Lactate and D-Glucose, although different from those recorded in *C. pagurus* still suggest that EMF exposure is altering melatonin levels within both species.

Haemocytes in crustaceans are known to contain potent antibacterial compounds that work against both gram-positive and gram-negative bacteria and have been found to work in low concentrations (Chrisholm and Smith, 1992). Lobster THC at 12 h was significantly lower in the EMF exposed lobsters than in control. THC counts decline in the presence of pathogenic bacteria, and or during starvation in *H.americanus* (Stewart 1967). Le Moullac *et al.* (1998) also found a decrease in THC within *Penaeus stylirostris*, after exposure to severe hypoxia. Previous studies have also found that EMF exposure reduced THC of *Trichoplusia ni* larvae after 48 h exposure (Valadez-Lira *et al.,* 2017). The reduction in THC during EMF exposure suggests an impaired immune system and consequently a decreased ability to fend off bacterial attacks. THC levels were then found to significantly rise back in line with those under control conditions after 24 h exposure. An increase in THC typically indicates an increased immune response in crustaceans (Le Moullac and Haffner, 2000). As haematocytes have a crucial role in the immune defense of crustaceans, there is a concern that EMF could potentially affect European lobster immune-physiological responses. The lower concentrations detected in EMF exposed lobsters at 12 h, when compared to the control, suggests an impaired immune system resulting in a decreased ability to defend against pathogenic bacteria. The lower concentrations appeared to be temporary (restricted to the first 12 h of exposure), with an immunological response resulting in the significant increase in Haemocytes from 6 h to 24 h which suggests an increase in Haemocyte production to combat the stress of EMF exposure.

Increased EMF exposure during egg development highlighted significant changes in egg volume, with decreases in total volume noted. There were pronounced differences in egg development during EMF exposure between *H. gammarus* and *C. pagurus* despite both showing significant differences at the several sampling dates. *H. gammarus* eggs showed a significant increase in egg size compared to the control followed by a sharp decrease whereas *C. pagurus* showed the opposite. In *C. pagurus* a significant decrease was

detected over the first 4 stages before showing significant increases compared to the control as the eggs approached full development. This suggests different responses, potentially due to changes in egg osmotic ability. EMF exposure during development was found to impact lobsters to a greater degree than *C. pagurus*. Larval deformities were significantly higher in those under EMF exposure, which coincides with an increase in deformities in other species when subjected to increased stress (Kurihara, 2008; Kawaguchi *et al.,* 2010; Gibson *et al*., 2011; Agnalt *et al.,* 2013). Hatched larvae were generally smaller across the majority of measured morphology, and an increase in failed larval fitness tests was observed. These studies highlight the significant effects of EMF exposure across all stages of *H. gammarus* life cycle. Further research is needed to fully assess the developmental impacts that EMF exposure has on the larvae as they progress through to adulthood.

Summary

"The early sailors knew their maps were not perfect, but imperfect maps were better than no map at all…." (Gosling and John, 1999)

The study of the effects of electromagnetic fields on marine species is relatively novel with only a handful of studies conducted, and even fewer on marine benthic species. The lack of data, and the high variability of the EMF strengths around deployments makes any work in this field problematic. With energy companies suggesting low values are expected around subsea power cables and scientists using strengths orders of magnitude higher in research, there is a real need for reliable data to be obtained. As each site will have different operating voltages, amps, cable configurations, number of turbines etc. the resulting fields will all vary in size and strength. This must be taken into account and future research would be best to focus on site specifics. Many argue that cable burial will solve EMF emission problems, and indeed many energy companies bury cables down to depths of around 1m (Ardelean and Minnebo. 2015). This will dampen the EMF on the seabed surface by way of increasing the distance from the cable, however EMF can penetrate all but a few materials, such as lead, and as such will still penetrate through the sediment. Cable burial does not solve the problems of EMF emission, instead it transfers the problem from surface organisms to those living within the benthos. This research

provides a significant amount of novel data which can hopefully begin to fill some of the gaps in our knowledge about these two species and the impacts of subsea power cables. This research has proven that EMF exposure causes behavioural and physiological impacts on both *C. pagurus* and *H. gammarus*. As both species have been shown to be of high ecological and economical importance, the potential impacts on these species needs to be addressed. Further research is needed to determine the long-term effects of EMF exposure on individuals, the effects of intermittent exposure, and the potential impacts EMF exposure will have on the various other species found around subsea power cables around the world. The impacts are not just limited to offshore deployments, through the need for export cables to transmit electricity, increased boating associated with maintenance and repair, and the impacts of construction and decommissioning the impacts are far reaching, including a variety of environmental stressors throughout a variety of habitats. The discovery of juvenile *C. pagurus* inhabiting holdfasts (Scott *et al.*, submitted) is an important factor that must be included in EIAs as part of the licensing for kelp harvesting. The potential loss of this important habitat due to harvesting for biofuels, laying of subsea power cables, creation of harbours etc. must be avoided where possible. Further research is required to investigate the larval settlement of *C. pagurus*, this information is lacking in the literature and would help with stock recruitment in addition to shedding light on habitat requirements of this species. With black spot occurrence shown to increase during immunocompromised individuals, the rapid increase of MREDs worldwide should be of major concern.

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